



VIA OVERNIGHT MAIL and VIA E-MAIL and VIA FACSIMILE

July 27, 2006

Mr. Floyd A. Landis
23356 Bishop Road
Murrieta, CA 92542

Michael P. Rutherford, Esq.
1435 Stuart Street
Denver, CO 80204

RE: UCI File No. 29/06
Tour de France, July 20, 2006
Sample #995474

Board of Directors

Ralph W. Holt, MD
Chair

Richard W. Cohen, MD
Vice Chair

Betsy Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Therese Breen, J. Ann Allen

John Fanczy, MD, PhD, MPH

Andrew Mecca, DrPH, MPH

Annexa Solomon, DrPH

Dear Mr. Landis:

Your urine sample collected at the Tour de France on July 20, 2006, was sent to the WADA accredited laboratory at Chateau-Malabry, France, ("the Laboratory") for analysis. The Laboratory has reported that your A sample contains an elevated testosterone/epitestosterone (T/E) ratio at a prohibited level of greater than 4:1. Additionally, the laboratory performed a Carbon Isotope Ratio (CIR) analysis on your sample and reported the result positive for exogenous testosterone or its precursors. The World Anti-Doping Agency's Prohibited List, adopted by both the USADA Protocol for Olympic Movement Testing ("Protocol") and the Union Cycliste Internationale ("UCI") Anti-Doping Rules, lists testosterone and its precursors as prohibited substances in the class of anabolic androgenic steroids.

I have enclosed the July 26, 2006, letter from UCI and the documents attached to that letter, including the Laboratory's report, referring your case. On July 26, 2006, USA Cycling forwarded the UCI documents to USADA and in accordance with the rules requested that USADA handle the adjudication of this matter. I have also enclosed a copy of this correspondence from USA Cycling to USADA and USADA's response of today to USA Cycling formally accepting the handling of your case.

As referenced in the UCI letter dated July 26, 2006, in accordance with UCI rules you must immediately request, without delay, the B sample analysis or the B sample analysis will be deemed waived and the laboratory results conclusively established. In accordance with UCI rule 194, the request for the analysis of the B sample must be made no more than five (5) working days from July 26, 2006, the date of the UCI letter. Please inform me immediately in writing by fax at 719-785-2028 if you elect to have the B opening and analysis performed and we will notify the Laboratory and UCI of your request and attempt to schedule the date for the B analysis. You also

United States Anti-Doping Agency

1330 Quail Lake Loop, Suite 260, Colorado Springs, CO 80906 ■ Tel 719.785.2000 ■ Fax 719.785.2001

usada@usantidoping.org ■ www.usantidoping.org

GDC0001

have the right to attend the B sample opening and analysis if requested (which may take many hours, over multiple days).

Further, if you choose not to request the B sample analysis, USADA will forward your case immediately to a panel of the independent USADA Anti-Doping Review Board, as set forth in the USADA Protocol, for its consideration. Under the USADA Protocol and the UCI Anti-Doping Rules, the finding of a prohibited substance or method in an athlete's sample constitutes a doping violation. If it is ultimately determined that this is your first doping violation, a sanction may be imposed that will include disqualification of your competitive results achieved on and subsequent to July 20, 2006, the day your sample was collected, and up to a two year period of ineligibility.

Additionally, you have the right, at this time, to accept a "provisional suspension." By accepting a "provisional suspension," you will be immediately suspended from competing in all competitions under the jurisdiction of UCI, USA Cycling, and the United States Olympic Committee ("USOC"), or any of these entities' clubs, member associations or affiliates, until your case is deemed not to be a doping offense, you accept a sanction, you fail to contest this matter, or a hearing has been held in this matter. If you choose to accept this "provisional suspension," the time served under the "provisional suspension" will be deducted from any period of ineligibility that you might receive beginning on the date you accept the "provisional suspension" and notify USADA of such acceptance. If you do not to execute and return this "provisional suspension," any period of ineligibility you might receive will begin on the date of your acceptance of the sanction or on the date of the arbitration hearing panel's decision.

If you execute and return the "provisional suspension," USADA will give notice to the USOC, UCI, and USA Cycling of your acceptance of the "provisional suspension." Your decision to accept this "provisional suspension" is purely optional. You do not have to accept this "provisional suspension" in order to proceed with your case. If you are willing to accept a "provisional suspension," please inform us in writing immediately, by executing and returning the attached USADA Acceptance of Provisional Suspension Form.

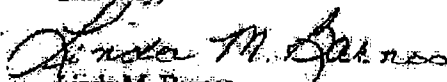
If you intend to compete in any protected competitions, USADA has the right under its Protocol, Section 13, to expedite this matter to final resolution prior to the protected competition.

Also of importance, you are still subject to testing pending the outcome of this matter.

USADA will not publicly disclose or comment on the specifics of your test results until your case has been resolved. By copy of this letter, USADA is notifying USA Cycling and the USOC of your test results and requests that these organizations not comment publicly concerning this information until disclosed as provided in the USADA Protocol.

Enclosed for your reference are copies of the USADA Protocol, UCI Anti-Doping Rules and the World Anti-Doping Code, which set forth the administrative procedures followed for positive and elevated tests and other alleged anti-doping rule violations. You may also wish to contact John Ruger, the USOC Athlete Ombudsman who is completely independent of USADA, or your own personal attorney, for assistance or further information. Mr. Ruger may be reached at One Olympic Plaza, Colorado Springs, CO, 80909, by telephone at (888)-ATHLETE, by fax at (303) 444-6626 or by e-mail at John.Ruger@usoc.org, or at www.888athlete.org.

Sincerely,



Linda M. Barnes
Testing Results Manager

cc: Sean Petty, USA Cycling (w/o encls.)
Gary Johansen, USOC Deputy General Counsel
Jim Scherr, USOC Chief Executive Officer
Christain Varin, UCI Anti-Doping Services (w/o encls.)

Enclosures: Correspondence from UCI to USA Cycling including:
Doping Control Form
Laboratory Report of Analysis
Correspondence from USA Cycling to USADA
Correspondence from USADA to USA Cycling
UCI Anti-Doping Rules
World Anti-Doping Code
WADA List of Prohibited Substances
USADA Protocol
USOC Anti-Doping Policies.

31/07/2006 20:45 914363329

07/31/2006 12:39 FAX 7552001

MASONS BUXEDA MENCHE

FAG. 04/05
07/07/2006

31/07/2006 19:06 914363329

MASONS BUXEDA MENCHE

FAG. 01/02

FLOYD LANDIS

1. To:
USA CYCLING
To Mr Steve Johnson
By fax +1 719 783 2023 +1 719 866 4628
2. Copy:
(i) INTERNACIONAL CYCLING UNION (UCI)
To Mr Christian Vain
By fax +41 24 468 5312
(ii) USADA
To Mr Travis Tygart
By fax +1 719 785 2001
(iii) LNDP - Laboratoire National de Dépistage du Dopage
To Mr Jacques de Cessart
By fax +33 1 4660 5017
(iv) PHONAK HEARING SYSTEMS
To Mrs Monica Zuercher
By fax +41 55 2547011

31st July 2006

Dear Sir:

I have received on the 26th July from my legal PHONAK HEARING SYSTEMS copy of a letter sent by UCI that same day concerning my testing positive (T/F) in the Tour de France on the eve of the 26th July.

By means of this letter:

- (i) I formally require the B sample analysis in accordance with articles 191 and following of the Anti-Doping Rules (ADR).
- (ii) I appoint as my legal representatives, Mr Luis Saez Hernández and Mr José M. Buxeda Malaterra, lawyers in Madrid (Spain). I request that any formal communication concerning this issue is sent to the latter (Mr José M. Buxeda, MASONS BUXEDA MENCHEN ABOGADOS, Calle José Ortega y Gasset, nº 25, 28006 - Madrid, T +34 914363329, F +34 914363729, e-mail: masonsbuxeda@masonsbuxeda.com). According to article 193 ADR, they will attend the opening of sample B.
- (iii) I appoint as the person that will attend the sample B analysis on my behalf, Dr Deuse de Roer, Department of Clinical Chemistry, University Hospital Maastricht.
- (iv) I require a copy of the complete analysis report for sample A that should be sent to my legal representatives as soon as possible.

GDC0004

31/07/2006 20:45 914353329

MASONS BUKEDA MENCHE

PAG. 05/05
0000/000

07/31/2009 12:30 FAX 7852001

31/07/2006 19:06 914353329

MASONS BUKEDA MENCHE

PAG. 02/02

-2-

(v) I require from the medical services of the UCI a thorough endocrinological study.
to be carried out in a specialized medical center in Europe and the United States.

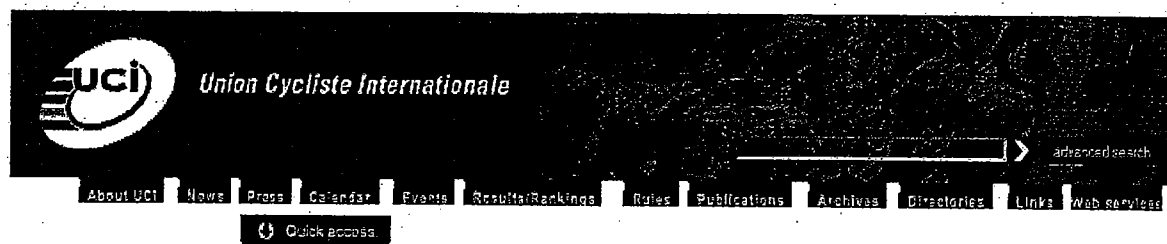
Yours sincerely,



31/7/06

Flavio L. Mendez

License: 0021572 - UCI Code: UBA 19751014



Français Printable version

News -> Latest news

05/08/2006: Press release : The adverse analytical finding from Floyd Landis is confirmed[News](#)[Latest news](#)[Archives](#)[Press releases](#)[Road](#)[Track](#)[Mountain bike](#)[Cyclo-cross](#)[BMX](#)[Trials](#)[Indoor Cycling](#)[Cycling for All](#)[Olympic Games](#)[Health and
anti-doping](#)[Newsletters](#)

The UCI communicates that the analysis of the sample B of Floyd Landis's urine has confirmed the result of an adverse analytical finding notified by the Anti-doping laboratory of Paris on 26th July, following the analysis of the sample A.

In accordance to the Anti-doping rules, the Anti-doping Commission of the UCI will request that the USA Cycling Federation open a disciplinary procedure against the rider.

UCI Press Service

[Top](#)

Copyright © 1997-2007 Union Cycliste Internationale. All rights reserved.

GDC0006

HOWARD L. JACOBS
LAW OFFICES OF HOWARD L. JACOBS
5210 Lewis Road, Suite 5
Agoura Hills, CA 91301
(818) 292-8735

Attorneys for Floyd Landis

UNITED STATES ANTI-DOPING AGENCY
INDEPENDENT ANTI-DOPING REVIEW BOARD

In The Matter Of
FLOYD LANDIS

SUBMISSION TO USADA INDEPENDENT ANTI-DOPING REVIEW BOARD

I. INTRODUCTION

This matter arises from the alleged positive drug test of Floyd Landis at the Tour de France on July 20, 2006. Mr. Landis allegedly tested positive for exogenous testosterone. Floyd Landis vehemently denies the allegations being made in this case.¹

This submission is made without access to complete documentation, and without access to any documents that would be required in any longitudinal study of

¹ While USADA may have redacted the name "Floyd Landis" in the documents provided to this Review Board, Landis will not participate in the charade that this is a confidential proceeding for two primary reasons: (1) given the improper leak and press statements by the UCI, the entire free world is aware that Floyd Landis, the 2006 Tour de France Champion, provided the only positive urine sample that is being pursued through disciplinary proceedings as a result of the 2006 Tour de France; and (2) the August 30, 2006 cover letter from USADA to the Review Board, which states "Re: UCI File No. 29/06, Tour de France, July 20, 2006," destroys any confidentiality in this specific proceeding given point (1) above.

testosterone/epitestosterone values. This submission should not be considered in any respect to be a complete recitation of the defenses that may be offered by or on behalf of Floyd Landis, and Floyd Landis specifically reserves the right to make any submission to any adjudication body in connection with these false charges. That being said, it is submitted that the documentation that has been provided to date does not meet the requisite positivity criteria as established by the World Anti-Doping Agency ("WADA"). Specifically, the following will be established:

1. The carbon isotope results do not satisfy the WADA positivity criteria, in that:
 - a. Only one of four metabolites tested clearly exceeds the 3‰ threshold provided by WADA;
 - b. The measurement value that is the best indicator of exogenous testosterone usage in urine proves that Floyd Landis did not use testosterone; and
 - c. All of the 5 α -Androstanediol ¹³C-values reported by LNDD are inaccurate.
2. Absent a positive CIR result, there is no case to answer under the WADA TECHNICAL DOCUMENT TD2004EAAS.

As such, there is no case to answer, and the proceedings should be dismissed by this Anti-Doping Review Board ("ADRB").

II. SUMMARY OF CARBON ISOTOPE RATIO RESULTS

USADA brings this case primarily based upon the carbon isotope ratio results ("CIR")², which USADA alleges provides evidence of the use of exogenous testosterone. A good summary of the CIR theory is provided at Maitre et al., Urinary Analysis of Four Testosterone Metabolites and Pregandiol by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry After Oral Administration of Testosterone, 28 Journal of Analytical Toxicology (Sept. 2004) [attached hereto as Exhibit 1]:

"IRMS allows measurements of slight differences in the carbon isotope ratio (¹³C/¹²C) of the exogenous and endogenous testosterone. Synthetic testosterone is produced from precursors derived from plants with low ¹³C content, whereas the ¹³C and ¹²C content in the natural endogenous form depends on the isotopic carbon composition of the food diet and is influenced by additional effects of human biological processing."

Carbon isotope ratios are expressed in terms of delta units per mil. Maitre went on to describe this calculation as follows:

The symbol δ is the standard notation for expressing carbon isotope ratios. It is defined as parts per thousand deviation of isotopic compositions from that of Pee Dee Belemnite (PDB), and is calculated according to:

$$\delta^{13}\text{C}/\text{‰} = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{standard}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}}$$

Once the $\delta^{13}\text{C}/\text{‰}$ value for the testosterone metabolites is calculated, the positivity criteria mandated by WADA requires that this value be compared between metabolites that are believed to be affected by exogenous testosterone use and those metabolites that are not so affected. See WADA Technical Document TD2004EAAS (attached hereto as Exhibit 2), p.3:

"3. Isotope ratio mass spectrometry:

² This CIR method is also referred to as Isotope Ratio Mass Spectrometry, or "IRMS."

When a parameter of the steroid profile indicates a need to further study, its $^{13}\text{C}/^{12}\text{C}$ value expressed in delta units per mil (‰) or that of its metabolites will be measured and compared to that of urinary reference steroids within the sample not affected by administration. Depending upon the nature of the endogenous steroid suspected to have been administered, the metabolites analysed could be ... androsterone, etiocholanolone, the androstane diols ... while the urinary reference steroid usually analysed by the Laboratories is one of, pregnanediol ... or 11-ketoetiocholanolone.”

Here, the French Lab (LNDD) that analyzed the Landis “A” and “B” samples tested for and calculated the ‰ values for the following testosterone metabolites that are affected by exogenous testosterone administration: androsterone, etiocholanolone, and the androstane diols (5 α -Androstane diol³ and 5 β -Androstane diol⁴). LNDD also tested for and calculated the ‰ values for the following testosterone metabolites that are not affected by exogenous testosterone administration: pregnanediol (specifically, 5 β -pregnanediol⁵) and 11-ketoetiocholanolone⁶. Without conceding the accuracy of the data, LNDD calculated the following values, expressed as corrected and uncorrected values:

For the “A” sample [see Document package, p. USADA 0185]:

| | True Value | Corrected Value |
|------------------|------------|-----------------|
| Androsterone | -27.71 | -25.05 |
| Etiocholanolone | -26.43 | -23.63 |
| 5 α Adiol | -32.12 | -27.72 |
| 5 β Adiol | -28.82 | -23.73 |
| | | |
| 11Ketoetio | -24.10 | -21.06 |

³ Also referred to as 5 α Adiol.

⁴ Also referred to as 5 β Adiol.

⁵ Also referred to as 5 β Pdiol.

⁶ Also referred to as 11-Ketoetio.

| | | |
|-----------------|--------|--------|
| 5 β Pdiol | -26.61 | -21.58 |
|-----------------|--------|--------|

For the "B" sample [see Document package, p. USADA 0351]:

| | True Value | Corrected Value |
|------------------|------------|-----------------|
| Androsterone | -27.93 | -25.29 |
| Etiocholanolone | -26.58 | -23.80 |
| 5 α Adiol | -31.88 | -27.43 |
| 5 β Adiol | -28.79 | -23.69 |
| | | |
| 11Ketoetio | -24.75 | -21.78 |
| 5 β Pdiol | -26.16 | -21.05 |

The final step in the analysis of the positivity criteria is stated in WADA

Technical Document TD2004EAAS (attached hereto as Exhibit 2), p.3, as follows:

"The results will be reported as consistent with the administration of a steroid when the $^{13}\text{C}/^{12}\text{C}$ value measured for the **metabolite(s)** differs significantly i.e. by 3 delta units or more from that of the urinary reference steroid chosen. In some *Samples*, the measure of the $^{13}\text{C}/^{12}\text{C}$ value of the urinary reference steroid(s) may not be possible due to their low concentration. The results of such analysis will be reported as "inconclusive" unless the ratio measured for the metabolite(s) is below -28‰ based on non-derivatized steroid."

In this case, LNDD calculated this difference for all four testosterone metabolites tested. In so doing, LNDD compared the measures of androsterone and etiocholanolone to the urinary reference steroid 11-Ketoetio; and compared the measures of 5 α Adiol and 5 β Adiol to the urinary reference steroid 5 β Pdiol. For reasons that are not stated, LNDD only calculated these measured differences based on corrected values (for sake of

completeness, the measured differences for the true values are provided here as well).

The measured differences are as follows:

For the "A" sample [See Document Package, pp. USADA 0185-0186]⁷:

| | True Measurement | Corrected Measurement |
|------------------------------|------------------|-----------------------|
| Etiocholanolone – 11Ketoetio | -2.33‰ | -2.58‰ |
| Androsterone – 11Ketoetio | -3.61‰ | -3.99‰ |
| 5βAdiol - 5βPdiol | -2.21‰ | -2.15‰ |
| 5αAdiol - 5βPdiol | -5.51‰ | -6.14‰ |

For the "B" sample [See Document Package, pp. USADA 0351-0352]⁸:

| | True Measurement | Corrected Measurement |
|------------------------------|------------------|-----------------------|
| Etiocholanolone – 11Ketoetio | -1.83‰ | -2.02‰ |
| Androsterone – 11Ketoetio | -3.18‰ | -3.51‰ |
| 5βAdiol - 5βPdiol | -2.63‰ | -2.65‰ |
| 5αAdiol - 5βPdiol | -5.72‰ | -6.39 ‰ |

III. THE CARBON ISOTOPE RESULTS DO NOT SATISFY THE WADA POSITIVITY CRITERIA

A. THE WADA POSITIVITY CRITERIA MUST BE READ AS REQUIRING THAT THE ¹³C/¹²C δ VALUE MEASURED FOR ALL METABOLITES

⁷ According to the LNDD documents themselves, these figures have a huge measure of uncertainty of ±0.8‰.

⁸ According to the LNDD documents themselves, these figures have a huge measure of uncertainty of ±0.8‰.

TESTED DIFFERS SIGNIFICANTLY, WHICH CRITERIA IS NOT MET IN THIS CASE

The WADA Positivity criteria, as mentioned above, requires a showing that “¹³C/¹²C value measured for the **metabolite(s)** differs significantly i.e. by 3 delta units or more from that of the urinary reference steroid chosen.” This requirement must be read as requiring that **all metabolites tested** differ significantly as described, i.e. by 3 delta units or more from the urinary reference standard chosen. Such a reading was confirmed in 2006 by the WADA-accredited laboratory in Lausanne:

“According to the technical document of the WADA Laboratory Committee, an athlete would be reported as consistent with the administration of a steroid when the ¹³C/¹²C-value measured for the **metabolites** differs significantly, i.e. by 3.0‰ or more from that of the urinary reference steroid chosen.” Baume et al., Use of Isotope Ratio Mass Spectrometry to Detect Doping with Oral Testosterone Undecanoate: Inter-Individual Variability of ¹³C/¹²C Ratio, Steroids 2006, at p. 6 [attached hereto as Exhibit 3]

In this case, it is clear that the Landis sample does not meet this positivity criteria, as only one of four metabolites tested clearly exceeds the 3‰ example provided by WADA (a second metabolite, measured at -3.51‰ ±0.8‰ on the “B” sample, cannot be said to exceed this threshold). For these reasons, the CIR results do not support a finding of exogenous testosterone use.

Landis submits that this criteria must be read as requiring that **all metabolites** tested exceed this threshold to declare the CIR test as positive. However, at worst, this criteria is vague and ambiguous, as the drafters – WADA – used the incredibly poor and imprecise description “¹³C/¹²C value measured for the **metabolite(s)**.”⁹

It is well settled law that ambiguities in a document or contract must be construed

⁹ Which description was clarified by Baume, supra, as requiring all tested metabolites to be positive under this criteria.

against the drafter of the document. See, e.g., 2 Restatement Contracts, 2d, § 206, p-105 [“In choosing among the reasonable meanings of a promise or agreement or a term thereof, that meaning is generally preferred which operates against the party who supplies the words or from whom a writing otherwise proceeds.”]; United States v. Seckinger, 397 U.S. 203, 216 (1970) [“our interpretation adheres to the principle that, as between two reasonable and practical constructions of an ambiguous contractual provision, such as the two proffered by the Government, the provision should be construed less favorably to that party which selected the contractual language. This principle is appropriately accorded considerable emphasis in this case because of the Government’s vast economic resources and stronger bargaining position in contract negotiations.”]¹⁰; USA Shooting & Q./International Shooting Union (UIT) (CAS 94/129) [““The fight against doping is arduous, and it may require strict rules. But the rule-makers and the rule-appliers must begin by being strict with themselves. Regulations that may affect the careers of dedicated athletes must be predictable ... They should not be the product of an obscure process of accretion.”]; USOC et al. v. IOC et al. (CAS 2004/A/725) [“The rationale for requiring clarity of rules extends beyond enabling athletes in given cases to determine their conduct in such cases by reference to understandable rules. As argued by Appellants at the hearing, clarity and predictability are required so that the entire sport community are informed of the normative system in which they live, work and compete, which requires at the very least that they be able to understand the meaning of rules and the circumstances in which those rules apply.”].

¹⁰ The only basis for the application of the UCI anti-doping regulations, and the WADA Technical Document that WADA and the UCI will assert is incorporated as binding in this case, is the contractual relationship between the parties. The analogy of the Government’s vast economic resources and stronger bargaining power is particularly apt in the context of athletes vs. anti-doping authorities.

Therefore, this positivity criteria must be read to mean that the $^{13}\text{C}/^{12}\text{C}$ δ value measured for the all metabolites tested differ significantly (i.e. by 3 delta units or more from that of the urinary reference steroid chosen). In addition to being required by settled law, such a reading of this positivity criteria makes sense: if an athlete were to take synthetic testosterone, and if that synthetic testosterone would cause a significant difference in the measurement of $^{13}\text{C}/^{12}\text{C}$ for one testosterone metabolite when compared to a urinary reference, then one should expect like or similar changes for all such metabolites tested. Simply stated, synthetic testosterone should not selectively affect these metabolites.

Furthermore, any notion that WADA intended otherwise, or that the WADA-accredited laboratories clearly understood that this positivity criteria would only require a showing of a single metabolite as exceeding the threshold, is easily dismissed by the following published statement by the WADA-accredited laboratory in Lausanne, which statement shortly post-dates the effective date of the WADA Technical Document TD2004EAAS:

“What are the IRMS criteria to determine endogenous T ingestion, that is, does all the measured T metabolite $\delta^{13}\text{C}$ -values or does only one have to be superior to 4‰.” See Maitre, Urinary Analysis of Four Testosterone Metabolites and Pregandiol by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry After Oral Administration of Testosterone, 28 Journal of Analytical Toxicology (Sept. 2004) [attached hereto as Exhibit 1].

If even the WADA-accredited laboratories were asking this question, then WADA can hardly claim that its laboratories understood otherwise. Absent a clarification by WADA, which clarification never occurred, this criteria must therefore be read as requiring that all the measured T metabolite $\delta^{13}\text{C}$ -values must show significant differences. Such a

reading is also consistent with the 2006 interpretation of the WADA Technical Document by the WADA-accredited laboratory in Lausanne (See Baume, supra).

In this case, it is clear that the Landis sample does not meet this positivity criteria, as only one of four metabolites tested clearly exceeds the 3‰ example provided by WADA (a second metabolite, measured at $-3.51‰ \pm 0.8‰$ on the "B" sample, cannot be said to exceed this threshold). For these reasons, the CIR results do not support a finding of exogenous testosterone use, and must be considered as negative.

B. THE MEASUREMENT VALUE THAT IS THE BEST INDICATOR OF EXOGENOUS TESTOSTERONE USAGE IN URINE PROVES THAT FLOYD LANDIS DID NOT USE TESTOSTERONE

Additional findings from the CIR results further undermine the erroneous conclusion that those results support a finding of exogenous testosterone use. Published research by WADA-accredited laboratories shows that the measurement 5 β Adiol - 5 β Pdiol is a better indicator of exogenous testosterone usage than other metabolite measurements, and should allow for longer detection periods of exogenous testosterone than the other metabolites.

See Maitre, Urinary Analysis of Four Testosterone Metabolites and Pregandiol by Gas Chromotography-Combustion-Isotope Ratio Mass Spectrometry After Oral Administration of Testosterone, 28 Journal of Analytical Toxicology (Sept. 2004)

[attached hereto as Exhibit 1]:

"This paper describes the time courses of isotopic ratio values in urine of androsterone (Andro), etiocholanolone (Etio), 5 α -androstanediol (5 α A), 5 β -androstanediol (5 β A), and the endogenous reference 5 β -pregnanediol (5 β P) in the frame of an excretion study following oral ingestion of testosterone initially and 13 h later by a healthy, male Caucasian volunteer ...

“Similarly to the T/E ratio, the $\delta^{13}\text{C}$ -values of the four T metabolites decrease rapidly after T administration with a difference of about 5‰ with respect to the endogenous reference 5 β P ...

“our results suggest that measurements of 5 β -androstanediol δ -values allow the detection of a testosterone ingestion over a longer period than other T metabolites $\delta^{13}\text{C}$ -values.”

Therefore, if an athlete used exogenous testosterone, his measured difference 5 β Adiol - 5 β Pdiol should be greater than his measured difference 5 α Adiol - 5 β Pdiol. In the Landis sample, this is not even close to the case: LNDD reported the following corrected values:

For the “A” sample:

5 β Adiol - 5 β Pdiol: -2.15‰
5 α Adiol - 5 β Pdiol: -6.14‰

For the “B” sample:

5 β Adiol - 5 β Pdiol: -2.65‰
5 α Adiol - 5 β Pdiol: -6.39‰

Had Landis used exogenous testosterone, the Maitre publication indicates that his 5 β Adiol - 5 β Pdiol should be at or greater than -6‰, given the measurement of 5 α Adiol - 5 β Pdiol. At a minimum, one would expect the 5 β Adiol - 5 β Pdiol to exceed the threshold of 3‰, which it does not. The only conclusion that can be drawn from the fact that the 5 β Adiol - 5 β Pdiol measurement is well below the threshold, when the WADA-accredited laboratories state that this measurement is the **best indicator** of exogenous testosterone administration, is that Floyd Landis did not use exogenous testosterone.

C. THE 5 α -ANDROSTANEDIOL ^{13}C -VALUES REPORTED BY LNDD ARE INACCURATE

With the 5 α Adiol - 5 β Pdiol measurement being significantly at odds with all of the other measurements in this case, the Review Board must consider the cause of this disparate measurement, which is inconsistent with every other measurement in the CIR portion of the analysis. It is submitted that the explanation for this erroneous measurement can be found in LNDD's calculation of incorrect values for 5 α Adiol, as evidenced by an examination of the negative control urine $\delta^{13}\text{C}$ -values for that metabolite. Simply put, LNDD's $\delta^{13}\text{C}$ -values for 5 α Adiol for the negative control urine show that their equipment was, for some unexplained reason, measuring excessively low $\delta^{13}\text{C}$ -values for 5 α Adiol.

Published data provides guidance for expected $\delta^{13}\text{C}$ -values for 5 α Adiol for negative control urines and for positive control urines. See Aguilera et al., Performance Characteristics of a Carbon isotope Ratio Method for detecting Doping with Testosterone Based on Urine Diols: Controls and Athletes with Elevated Testosterone/Epitestosterone Ratios, 47 Clinical Chemistry 292, 296 Table 3 (2001) [attached hereto as Exhibit 4], showing that mean $\delta^{13}\text{C}$ -values for 73 negative control urines for 5 α Adiol was -26.35‰, with a maximum of -24.55‰ and a minimum of -27.89‰. See also, Maitre, Urinary Analysis of Four Testosterone Metabolites and Pregandiol by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry After Oral Administration of Testosterone, *supra*, showing that mean $\delta^{13}\text{C}$ -values for negative control urines for 5 α Adiol was -24.3‰ (± 0.4 ‰). In contrast to those figures, LNDD measured $\delta^{13}\text{C}$ -values for negative control urines for 5 α Adiol in the Landis case of -28.40‰ on the "A" sample (See Document package, p. USADA 0185) and -28.31‰ on the "B" sample testing (See Document package, p. USADA 0351). These figures are inconsistent with reported

figures as shown above, and in fact, are more consistent with measurement or calibration error. See, e.g., Maitre, supra, reporting that mean $\delta^{13}\text{C}$ -values for **positive control urines** for 5 α Adiol was -28.4‰ ($\pm 0.5\%$). See also, Shackleton et al., Confirming Testosterone Administration By Isotope Ratio Mass Spectrometric Analysis Of Urinary Androstenediols, 62 Steroids 379, 383 (1997) [attached hereto as Exhibit 5] ["In our studies with the Chinese subjects, it can be stated that for the five individuals, none had androstenediol $\delta^{13}\text{C}\%$ values less than -28.3 during the control period."]

The LNDD readings for 5 α Adiol in the negative control urine are so low that they must be inaccurate. In fact, those readings look more like positive control urine values than negative controls. If the negative control urine readings for 5 α Adiol are excessively low, it must be the case that the LNDD readings of the Landis sample for 5 α Adiol are also excessively low and inaccurate, thus explaining the large difference in the 5 α Adiol - 5 β Pdiol measurement. As this measurement in the Landis sample is totally at odds with any of the other measurements as discussed above, it is submitted that the result must stem from laboratory error.

D. SUMMARY

As shown above, the WADA Positivity Criteria or CIR analysis of exogenous testosterone usage has not been met:

1. Whereas the WADA Positivity Criteria requires all four testosterone metabolites to provide clear evidence of testosterone usage, 3 of the 4 metabolites must be considered as negative;

2. The only testosterone metabolite that is even arguably positive under the WADA Positivity Criteria is the result of laboratory error and not the result of testosterone usage; and
3. The one metabolite that has been identified by the WADA-accredited laboratories as the best indicator of exogenous testosterone usage, and the longest-term indicator of exogenous testosterone usage, has been reported as negative.

Any one of these deficiencies would alone be sufficient to render the CIR result negative.

IV. ABSENT A POSITIVE CIR RESULT, THERE IS NO CASE TO ANSWER UNDER THE WADA TECHNICAL DOCUMENT TD2004EAAS

A negative CIR result in most cases mandates a dismissal of doping allegations of exogenous testosterone usage. However, in all cases other than a positive CIR Result, WADA Technical Document TD2004EAAS requires that a longitudinal study be performed. No such longitudinal study has been performed in this case, and no such longitudinal data has been provided to the athlete or to this Review Board. For this reason, there is no case to answer, and the case against Floyd Landis must be dismissed.

Doping charges cannot proceed against an athlete based upon an inconclusive/negative CIR test and a single T/E value. Furthermore, the single T/E analysis in this case is replete with fundamental, gross errors. Examples of these errors include:

1. Mismatched sample code numbers that do not belong to Floyd Landis (see, e.g., Document Package p. USADA 0288, alleged confirmation T/E

data on "B" sample, containing different sample number from that assigned to Floyd Landis; see also Document package USADA 0024, LNDD chain of custody documentation regarding receipt of sample, does not identify any sample numbers matching the code number for the Floyd Landis sample). Clinical laboratories making these types of gross errors could easily find themselves answering to a wrongful death lawsuit.

Simply stated, if LNDD cannot get the sample code number correct, how can they be trusted to accurately report quantitative test results?

2. Grossly inconsistent testosterone and epitestosterone samples from sequential tests on the Landis "A" sample:
 - a. See Document Package, pp. USADA 0212 and 0223, testing on Landis sample 995474, vial 10 aliquot (first "A" confirmation analysis), showing testosterone level of 172.23 ng/ml and epitestosterone level of 17.59 ng/ml; and showing corrected values of 127 ng/ml for testosterone and 13 ng/ml for epitestosterone;
 - b. Compare Document package, pp. USADA 0092 and 0101, vial 4 aliquot (second "A" confirmation analysis), showing testosterone level of 61.37 ng/ml and epitestosterone level of 5.20 ng/ml; and showing corrected values of 45.4 ng/ml for testosterone and 3.9 ng/ml for epitestosterone;
 - c. It must be accepted that two test results using the same method on the same urine and tested sequentially should not show three-fold differences in testosterone and epitestosterone. Such differences are

clear evidence of laboratory error, such that none of these results can be accepted as accurate.

“Where doubt has been created with regard to the test procedure, such doubt must go to the benefit of the athlete.” USA Triathlon v. S. Smith (CAS 99/A/241). The LNDD laboratory documents are replete with such gross errors and ineptitude that their results in this case cannot be seriously accepted as accurate. At a minimum, those laboratory errors must go to the benefit of the athlete, and must result in a finding that the T/E results are wholly unreliable.

V. CONCLUSION

For the foregoing reasons, it is submitted that there can be no case to answer, and that the charges against Floyd Landis must be immediately dismissed.

RESPECTFULLY SUBMITTED,

DATED:

LAW OFFICES OF HOWARD L. JACOBS

By: _____
Howard L. Jacobs
Attorneys for Floyd Landis

September 15, 2006

Terrence P. Madden
Chief Executive Officer
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906

Re: RE: UCI File No. 29/06
Tour de France, July 20, 2006
Sample #995474

Dear Terry,

The following members of the United States Anti-Doping Agency's Anti-Doping Review Board Panel met by teleconference on September 18, 2006, to review the above referenced matter.

This Panel considered the written information submitted to it and concluded that there was sufficient evidence of doping to proceed with the adjudication process as set forth in USADA's Protocol for Olympic Movement Testing.


Bernard Favaro, Esq., Chair

Fred Apple, Ph.D.

Robert Dimeff, M.D.

C. Ayotte, D. Goudreault, A. Lajeunesse, M. Cléroux, Y. Richard, A. Charlebois, J.-P. Couture
and A. Falciani

GC/C/IRMS and GC/MS in "Natural" Steroids Testing

Centre de recherche en santé humaine, INRS-Institut Armand-Frappier, Montréal

Introduction

Testing for the administration of natural steroids is a complex task requiring the identification and quantification of a number of parameters of the steroid profiles, one of which being the T/E¹ value. Complementary diagnostic information is gained from the determination of the urinary concentration of testosterone, epitestosterone, androsterone and etiocholanolone. By 1997, three different groups had proposed the GC/C/IRMS as a promising tool for the detection of the administration of testosterone (Aguilera et al., 1996; Becchi et al., 1994; Horning et al., 1997; Shackleton et al., 1997) and since then, others have described the values of the ¹³C/¹²C (δ ¹³C₁₀₀) of urinary androgens within the athletic populations and following the administration of DHT, epitestosterone or testosterone precursors such as DHEA.

In this paper, we present the results afforded by the analysis of urine samples collected following the administration of testosterone, androstenedione and DHEA as well as examples of the combined GC/MS and GC/C/IRMS individual "profiles" which have been proven to be useful in the investigation of putative doping cases, to discriminate inter alia, between the administration of a source of testosterone and 19-nortestosterone from a natural and systematic excretion of elevate T/E values or an increased endogenous norandrosterone production.

Experimental

"Androstenedione complex", DHEA and Nordione were purchased with an authorization from Health Canada (8572.090.98) from Price's Power International (International Nutrition and

¹ The T/E value is the corrected area ratio of both peaks.

Export, Newport News, Virginia 23608, USA). One capsule was administered to volunteers after verification of its content by GC/MS analysis. Urine samples were collected 24 hours before and up to one week after the administration and were prepared and analysed according to anabolic agents' procedure for the GC/MS steroid profiling. The preparation of the specimens for the GC/CIRMS was carried out according to a modification of the method proposed by Shackleton (1997) for the analysis of testosterone and precursors. The underivatized extracts of hydrolysed steroids (Ayotte et al., 1996) were analysed as such when the concentration of nortestosterone was greater than 30 ng/mL or following an HPLC fractionation for lower levels. The identity of the peaks was verified by GC/MS. Authentic standards were purchased from Steraloids Inc. (Wilton, NH 03086).

Results and discussion

Testosterone, androstenedione and DHEA:

The administration of testosterone and precursors, androstenedione and DHEA can be detected by the GC/MS determination of parameters of the urinary steroid profile that are abnormal when compared to the ranges of values normally found in human (Donike et al., 1983, 1993). The oral intake of androstenedione and DHEA was shown to transiently increase the excreted T/E value in females and, although not systematically, in some males (Bowers, 1999; Bory et al., 1998; Van Banno et al., 1998; Gault and Palonek, 1998; Uralis and Gillette, 1999; Lévésque and Ayotte, 1999; Lévésque et al., 1999). Other alterations of the urinary steroid profile, such as an abnormally high concentration of androstene and etiocholanolone, the presence of the characteristic hydroxylated metabolites glucuro- and sulfoconjugated, 6 α -hydroxyandrostenedione, 6 β -hydroxyandrostenedione for androstenedione and for DHEA, the increased excretion of 7 β -hydroxyandrostenedione with suppression of 16 α -hydroxyandrostenedione, served as basis for reporting positive findings (Lévésque and Ayotte, 1999; Lévésque et al., 1999). The disruption of the normal urinary profiles of androgens metabolites can be demonstrated by comparison with the described population reference ranges that are summarised in table 1 (Ayotte, 1997 and reference cited therein; Lévésque and Ayotte, 1999; Catlin et al., 1997; Geyer et al., 1997; Bauminger and Bowers, 1994). It also requires the

investigation of the athlete's previous or subsequent tests results in order to exclude the few individuals who naturally produce urine samples in which elevated T/E values are systematically measured. A systematic excretion of elevate T/E values reflects a normal condition, while sudden increase deviating from the athlete's norm, is consequent with the administration of source of testosterone, including the precursors (Ayotte, 1997; Geyer et al., 1997). The evaluation of some cases that we have made in the past years, based upon the variation of the individual's T/E values, are illustrated in Table 2.

Table 1: Description of the steroid profiles in the athletes' reference populations

| Parameter | Description (97.5%) | | Reference |
|--------------|---|--|---|
| | Male athletes | Female athletes | |
| T/E values | 4.2 (n=11000) 5.2 (n=5000) 6.5-7.0 (99%; n=22806) | 3.2 (n=4667) 6.3 (n=1700) | Lévésque and Ayotte, 1999 Geyer et al., 1997 Bauminger and Bowers, 1994 |
| T (ng/mL) | 5.6 (99%; n=3700) 106 ng/mL (n=9500) | 26.4 ng/mL (n=3740) 57 ng/mL (n=1700) | Catlin et al., 1997 Lévésque and Ayotte, 1999 |
| A (ng/mL) | 137 ng/mL (n=5000) 6703 ng/mL (n=9500) | 5170 ng/mL (n=4200) 6439 ng/mL (n=1700) | Geyer et al., 1997 Lévésque and Ayotte, 1999 |
| Etio (ng/mL) | 6689 ng/mL (n=5000) 5294 ng/mL (n=9500) | 4938 ng/mL (n=4200) 6107 ng/mL (n=1700) | Geyer et al., 1997 Lévésque and Ayotte, 1999 |

Table 2: Evaluation of elevate T/E values in athletes' samples

| Normally elevated cases | | T/E value | Remarks |
|-------------------------|-----------|-----------|------------------------------------|
| Mean | Variation | Range | |
| Male 1 | 4.8 | 23% | 2.9 to 6.9 (n=20) |
| Male 2 | 1.3 | 13% | 1.0 to 1.5 (n=11) |
| Male 3 | 5.8 | 25% | 3.1 to 8.7 (n=14) |
| Female 1 | 0.8 | 38% | 0.3 to 1.2 (n=16) |
| Female 2 | 1.1 | 43% | 0.5 to 2.1 (n=13) |
| Female 3 | 6.1 | 39% | 3.0 to 9.2 (n=11) |
| Positive cases | | | |
| Male 4 | 3.9 | 68% | 2.3 to 10.6 (n=8) |
| | 2.9 | 24% | 2.2 to 3.2 (n=7) |
| Male 5 | 1.8 | 70% | 0.9 to 7.3 (n=21) |
| | 1.5 | 22% | 0.9 to 2.2 (n=20) |
| Female 4 | 2.0 | 132% | 0.6 to 11.6 (n=16) |
| | 1.2 | 38% | 0.6 to 1.9 (n=14) |
| | | | Excluding two elevate test results |

Several groups have reported significantly changed carbon isotopic $^{13}\text{C}/^{12}\text{C}$ values (expressed as $\delta^{13}\text{C}/\text{‰}$) in the androgens metabolites excreted following the administration of testosterone (Aguilera et al., 1999, 2000, 2001), DHT, epitestosterone, DHEA (Shackleton et al., 1997, Ueki and Okano, 1999), and corticosteroids (Bourgonne et al., 2000). The methods described are based upon the comparison of the $\delta^{13}\text{C}/\text{‰}$ values of the urinary androgens metabolites to reference endogenous steroids, and products from earlier processes of the endogenous biosynthetic pathways. The urinary androgens metabolites most frequently analysed are androstosterone, etiocholanolone, 5 α - and 5 β -androstane-3 α ,17 β -diol, whereas the endogenous unaltered reference steroids are pregnanediol, pregnanetriol and cholesterol. The analysis of $\delta^{13}\text{C}/\text{‰}$ natural values amongst athletes did not indicate differences related to the nationality but to the diet (Aguilera et al., 1999, 2000, 2001; Shackleton et al., 1997, Ueki and Okano, 1999). To compensate for the different analytical methodologies and to allow for inter-laboratory comparison, it was proposed to use the ratio of the values of the androgens metabolites to those of the endogenous reference steroids in each sample (Shackleton et al., 1997).

19-nortestosterone and precursors, norandrostenedione and norandrostenediol:

The administration of 19-nortestosterone and of its precursors, 19-norandrostenedione and 19-norandrostenediol, results mainly in the excretion of 19-norandrostosterone (NA) and 19-noretiocholanolone (NE), mostly found in the glucuronidated form. The period during which the metabolites can be detected, is drastically reduced when the oral preparations are taken and the relative amounts of NA and NE can vary based upon the individual and the product taken (Engel et al., 1958; Massé et al., 1985; Schinzel, 1996; Kintz et al., 1999). The low excretion of endogenous norandrostosterone is normally not detected in human urine samples during routine doping control testing, with limits of detection of around 0.2 to 0.5 ng/mL, excepting specimens collected during pregnancy (Reznik et al., 1987). A more sensitive analytical technique such as the GC/HRMS, a larger volume of urine extracted and an extensive sample clean-up are needed to detect, identify and quantify endogenous NA, which in some male specimens was quantified at levels varying around 0.01 to 0.32 or 0.05 to 0.6 ng/mL, well below the limit of the IOC (Jennaneau et al., 1999; Le Bizac et al., 1999; Delemmin, 1999). In females, the level of NA is normally also very low, reaching a mean maximum value of 0.6 ng/mL during the ovulation

(Hennrichbach et al., 2000). The statistics collated by the IOC indicate that each year, are 0.24% of the A-samples analysed were reported to contain norandrostosterone, making norandrostosterone one of the anabolic agents most frequently found in athletes' samples along testosterone.

Natural Steroids $\delta^{13}\text{C}/\text{‰}$ values

We have measured the $\delta^{13}\text{C}/\text{‰}$ values of urinary steroids excreted in mixed athletes' same different nationalities and found them to be similar to those reported by others (table 3).

Table 3: Reference $\delta^{13}\text{C}/\text{‰}$ values measured in athletes' samples

| Steroid | n | Mean | Std dev. | $\delta^{13}\text{C}/\text{‰}$ | Range |
|-----------------|----|-------|----------|--------------------------------|----------|
| Androstosterone | 78 | -23.4 | | 1.6 | -19.9 to |
| Etiocholanolone | 78 | -23.3 | | 1.3 | -20.0 to |
| Pregnanediol | 56 | -24.9 | | 1.2 | -22.1 to |
| Pregnanetriol | 68 | -23.8 | | 1.5 | -20.8 to |
| Cholesterol | 68 | -24.2 | | 1.5 | -21.1 to |

Synthetic $\delta^{13}\text{C}/\text{‰}$ values

The GC/HRMS analysis of samples collected before and following the administration of testosterone and of a single oral dose of androstenedione and DHEA have shown that the content of the androgens metabolites differed significantly from their original value (stable the course of the study) and from those of the reference steroids, going to the depleted value measured in synthetic steroids. Examples of results are summarised in tables 4, 5 and in 6, 1, 2.

We are now using the combination of GC/MS and GC/HRMS to evaluate "testosterone" Examples of normally and abnormally elevated T/E excretion are presented in table 6.

Table 4: $\delta^{13}\text{C}/\omega$ values of urinary steroids modified by the administration of androstenedione.

| Androstenedione | α -adial | β -adial | Etio | A | Pd | Pl | Chol |
|-----------------|-----------------|----------------|-------|-------|-------|-------|-------|
| Vol. 1 | | | | | | | |
| Before | Mean | -24.4 | -24.3 | -24.6 | -24.3 | -25.5 | -23.8 |
| | Std dev | 1.7 | 0.5 | 0.3 | 0.1 | 0.3 | 0.5 |
| After | Mean | -31.4 | -32.9 | -33.7 | -31.7 | -25.3 | -24.1 |
| | Std dev | 2.6 | -3.2 | 1.9 | 2.4 | 0.5 | 0.8 |
| Difference | | -7 | -8.5 | -9.1 | -7.3 | 0.3 | 0.3 |
| Vol. 2 | | | | | | | |
| Before | Mean | -24.4 | -24.4 | -24.4 | -23.8 | -22.3 | -23.0 |
| | Std dev | - | - | 0.3 | 0.2 | 0.2 | 0.3 |
| After | Mean | -31.4 | -32.4 | -33.8 | -33.9 | -22.5 | -23.7 |
| | Std dev | 3.8 | 2.4 | 1.7 | 2.2 | 0.6 | 0.8 |
| Difference | | - | - | -9.4 | -10.1 | -0.2 | -0.6 |

Table 5: $\delta^{13}\text{C}/\omega$ values of urinary steroids modified by the administration of DHBA.

| DHBA | α -adial | β -adial | Etio | A | Pd | Pl | Chol |
|------------|-----------------|----------------|-------|-------|-------|-------|-------|
| Vol. 1 | | | | | | | |
| Before | Mean | -23.5 | -25.0 | -25.7 | -25.8 | -24.7 | -24.4 |
| | Std dev | 0.8 | 1 | 0.7 | 0.9 | 0.5 | 0.3 |
| After | Mean | -33.8 | -31.8 | -33.6 | -34.0 | -25.2 | -24.5 |
| | Std dev | 2.4 | 4.7 | 2.3 | 2.6 | 0.6 | 0.4 |
| Difference | | -10.3 | -6.8 | -7.9 | -8.3 | 0.5 | 0.1 |
| Vol. 2 | | | | | | | |
| Before | Mean | -23.3 | -25.8 | -25.8 | -24.8 | -24.9 | -23.9 |
| | Std dev | - | 1.1 | 0.9 | 0.8 | 0.5 | 0.6 |
| After | Mean | -32.6 | -33.7 | -35.7 | -34.1 | -24.8 | -24.5 |
| | Std dev | 2.6 | 3.1 | 1.2 | 2.1 | 0.8 | 1.1 |
| Difference | | - | -10.4 | -9.9 | -9.3 | 0.1 | -0.6 |

² The signal was too low to permit an accurate determination.

Table 6: Evaluation of testosterone cases using the combination of GC/MS and GC/CIRMS

| | T/E | Etio | A | Pd | Pl | A/Pd |
|---------------------------|------------------------|--------|--------|-------------|--------|---------------|
| Testosterone ¹ | 15.7 | -28.5 | -28.1 | -23.8 | -24.0 | 1.2 |
| (Variation %) | (32%) | (2%) | (2%) | (3%) | (3%) | (3%) |
| Athlete 1 (negative) | 4.2 ² (40%) | -22.6 | -23.7 | -23.7 | -23.8 | 1.0 |
| Athlete 2 (negative) | 6.7 ³ | -26.0 | -26.3 | -26.7 | -26.2 | 1.0 |
| | (23%) | (4%) | (3%) | (4%) | (0.3%) | |
| Athlete 3 (negative) | 6.1 ⁴ | -21.7 | -22.2 | -23.0 | -23.5 | 1.0 |
| | (25%) | (6.5%) | (3.8%) | (0.3%) | (6.1%) | |
| Athlete 4 (positive) | 38 | -31.3 | -31.9 | Cholesterol | -25.3 | A/Cholesterol |
| | | | | -25.7 | | 1.2 |

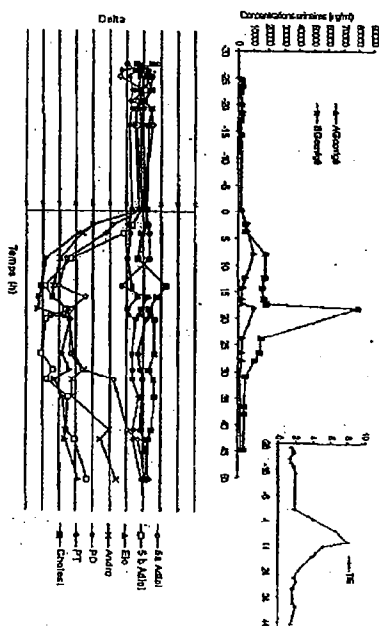


Figure 1: Variation of T/E values, corrected concentration of A and Etio and of $\delta^{13}\text{C}/\omega$ values following the administration of a single dose of androstenedione in male volunteer 1.

¹ n = 15 urine samples (Medical treatment: testosterone enanthate once per week, two weeks collection)
² T/E: n = 8 $\delta^{13}\text{C}/\omega$ single determination
³ T/E: n = 4 $\delta^{13}\text{C}/\omega$ single determination
⁴ T/E: n = 2 $\delta^{13}\text{C}/\omega$ n = 2

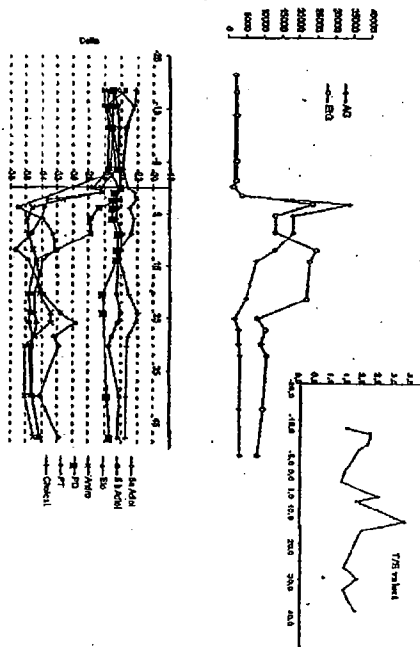


Figure 2: Variation of T/E values, corrected concentration of A and Etio and of $\delta^{13}\text{C}_{16}$ values following the administration of a single dose of DHEA (200 mg) in male volunteers³.

19-nortestosteroids

Twenty-five urine samples, analysed between 1992 to 1999, containing nortestosterone in amounts ranging from 35 to 11000 ng/mL, were re-analysed by GC/CIRMS and so were the urine samples collected after the administration of nortestosterone (commercial Nortestone). The $\delta^{13}\text{C}_{16}$ values measured in the excreted nortestosterone and nortestosterone were both significantly different than those of the reference steroids and of androstosterone and etiocholanolone, excepting of course, athletes' samples in which elevated T/E values were also measured.

Endogenous nortestosterone (around 2 ng/mL), excreted during the 12th and 14th weeks of pregnancy of two different persons, was analysed by GC/CIRMS following its isolation from a large volume of urine. The $\delta^{13}\text{C}_{16}$ values then measured were found to be similar to the other endogenous steroids, androstosterone, etiocholanolone, pregnanediol and pregnanetriol. The results are summarised in table 7.

Table 7: $\delta^{13}\text{C}_{16}$ values of nortestosterone in 1) nortestosterone positive samples 2) nortestosterone samples 3) pregnancy samples

| | $\delta^{13}\text{C}_{16}$ (mean values) | | | | | |
|--|--|-------|-------|-------|-------|-------|
| | NA | NR | A | Pd | Pr | NA/Pd |
| Nortestosterone ¹ | -31,1 | -31,5 | | -23,0 | -23,0 | 1,35 |
| Nortestosterone ² | -31,6 | -30,4 | -22,7 | -23,4 | -22,4 | 1,36 |
| Pregnancy 12 th week ³ | -23,6 | | -24,9 | -23,1 | | 1,02 |
| Pregnancy 14 th week | -23,0 | | -26,1 | -23,0 | | 1,00 |

Conclusion

The combination of GC/MS and GC/CIRMS has been proven useful to the evaluation of cases where the administration of "natural" steroids was suspected. The population reference range of the diagnostic parameters of both techniques are now well described. The ratio of the $\delta^{13}\text{C}_{16}$ values measured in the steroids metabolites to the unaltered reference steroids is the only way to compensate for the many diets and for the different methods that are currently used in the laboratories. Alone, absolute values associated with either synthetic or natural steroids cannot form basis of the decision, significant differences having reported for the reference population. The ratio of 1,1:1,0 proposed by Shackleton (1997) for testosterone seems also adequate for other steroids. The choice of the reference steroids must be made taking into account the alterations of the values that could be produced following the administration of precursors such as pregnenolone. When the concentration and the volume of specimen available permitted the injection of more than 20 ng, the ratios of the urinary synthetic nortestosterone to the unaltered reference steroids were always high since the contribution of natural nortestosterone can be virtually excluded. Natural values, similar to those of the reference steroids, were obtained during pregnancy.

¹ n = 25 urine samples

² Results of one excretion study. The mean value of commercial capsules: -32,7

³ One determination: extracted from 560 mL and 380 mL, respectively

Acknowledgements

The financial support of the Canadian Centre for Ethics in Sports (CCES), of the International Association of Athletic Federations (IAAF), the International Drug Testing and Management (IDTM) through their testing programmes is gratefully acknowledged. The skilled contribution of Alain Charlebois and Jean-Pierre Couture is invaluable. Jean-François Lévesque and André Lajeunesse received grants from the FCAR and from Sport Canada for which we are grateful.

Reference

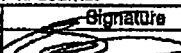
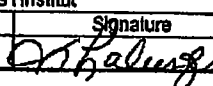
- Aguilera R., D.H. Catlin, M. Becchi, A. Phillips, C. Wang, R.S. Swerdloff, H.G. Pope and C.K. Hatton, *J. Chromatogr. B*, 727 (1999) 95
- Aguilera R., M. Becchi, H. Casabianca, C.K. Hatton, D. H. Catlin, B. Starcevic and H.G. Pope, *J. Mass Spectrom.*, 31 (1996) 169
- Aguilera R., T.E. Chapman and D.H. Catlin, *Rapid Commun. Mass Spectrom.*, 14 (2000) 2294
- Aguilera R., T.E. Chapman, B. Starcevic, C.K. Hatton and D.H. Catlin, *Clin. Chem.*, 47 (2001) 292
- Ayotte C., D. Goudreault and A. Charlebois, *J. Chromatogr.*, 687 (1996) 3
- Ayotte C., Evaluation of elevated testosterone/epitestosterone values in athletes' urine samples, *IAF New Studies in Athletics*, 12.2-3 (1997) p. 87 and reference cited
- Becchi M., R. Aguilera, Y. Fartzon, M. M. Flament, H. Casabianca and P. James, *Rapid Commun. Mass Spectrom.*, 8 (1994) 304
- Bosy T.Z., K. A. Moore and A. Poklis, *J. Anal. Toxicol.*, 22 (1998) 455
- Bourgogne E., V. Herrou, J.-C. Mathurin, M. Becchi and J. de Ceauritz, *Rapid Commun. Mass Spectrom.*, 14 (2000) 2343
- Bowers L.D., *Clin. Chem.*, 45 (1999) 293
- Donike M., in *Proceedings of the 10th Cologne Workshop on Dope Analysis*, 7th to 12th June 1992, M. Donike, H. Geyer, A. Goltzmann, U. Marek-Engelke, S. Rauth eds., Sport und Buch Strauss Ed. Sport, Köln, (1993) p. 47
- Donike M., K.-R. Barwald, K. Klostermann, W. Schanzer and J. Zimmermann, *Nachweis und exogenem Testosteron in Sport: Leistung und Gesundheit*, H. Heck, W. Hollmann, H. Litschke Rotts eds., Deutscher Arzte Verlag Köln, (1983) p.293
- Engel L., J. Alexander and M. Wheeler, *J. Biol. Chem.*, 231 (1956) 159
- Garle M. and B. Palonik, *Recent advances in doping analysis* (6), *Proceedings of the 10th Workshop on Dope Analysis*, (1998) p. 181
- Horning S., H. Geyer, M. Machnik, W. Schanzer, A. Hilker and J. Oefelmann, in *Recent Advances in Doping Analysis* (4), W. Schanzer, H. Geyer, A. Goltzmann, U. Marek-Engelke, eds., Sport und Buch Strauss Edition Sport, Köln (1997) p. 275
- Kintz P., V. Chimele et B. Ludes, *Acta Clin. Belg. Suppl.*, 1 (1999) 68
- Lévesque J.-F. and C. Ayotte, in *Recent Advances in Doping Analysis* (7), *Proceedings of the 17th Workshop on Dope analysis*, (1999) p. 169
- Lévesque J.-F. and C. Ayotte, *idem*, p. 213
- Massé, R.C. Laliberté, L. Tremblay and R. Dugal, *Biomed. Mass Spectrom.*, 12 (1985) 115
- Reznik Y., Herrou, M., Delennin, L., Lemaire, M., and Leymaire, P., *J. Clin. Endocrinol. Metab.* (1987) 1086
- Schanzer W., *Clin. Chem.*, 42:7 (1996) 1001
- Shackleton C.H.L., A. Phillips, T. Chang and Y. Li, *Steroids*, 62 (1997) 379
- Shackleton C.H.L., E. Roitman, A. Phillips and T. Chang, *Steroids*, 62 (1997) 665
- Ueki M. and M. Okano, *Rapid Commun. Mass Spectrom.*, 13 (1999) 2237
- Uriarte V.P. and P.A. Gillette, *J. Anal. Toxicol.*, 23 (1999) 357

Chaîne de possession
Laboratoire de contrôle du dopage

Réception ☐ Voûte réfrigérée arrière

| Date | Heure | No de connaissance | Nombre de colis |
|------------|-------|--------------------|-----------------|
| 15 Juin 06 | 10:50 | | 1 boîte |
| Sceaux | | | |
| | | | |
| | | | |

Remplir cette section pour les colis livrés en main propre seulement

| Identification du courrier | | Personnel de l'institut | |
|----------------------------|---|-------------------------|---|
| Nom (lettres moulées) | Signature | Nom (lettres moulées) | Signature |
| SM (205) |  | Micheline Laberge |  |

Entreposage avant enregistrement

| Colis entreposés par | Lieu d'entreposage | Date | Heure |
|----------------------|--------------------|---------|-------------|
| MM | EV-4 | 15-6-06 | 10:50 11h25 |

Codification et prélèvement

| Transfert au laboratoire | Date | Heure | Nombre d'échantillons |
|----------------------------|---------------------------------|-------|-----------------------|
| MM | 19-6-06 | 15h16 | 30 |
| Prélèvements effectués par | Procédures activées | | Ecart de codification |
| GPC | (1)(2)(3)(4)(5)(6)(7)(8)(9)(10) | | 06-07622 à 7651 |

Entreposage

| | Transféré par | Lieu d'entreposage | Date | Heure |
|----------------|---------------|--------------------|----------|-------|
| Prélèvements A | GPC | EV4 | 21-06-06 | 10h32 |
| Spécimens A | GPC | #17 | 21-06-06 | 10h32 |
| Spécimens B | MM | #17 | 17-6-06 | 18h47 |

Manipulations supplémentaires

| Code labo | Date | Heure | Initiales | Motif |
|-----------|-----------|----------|-----------|------------------------------|
| T045A | Récupéré | - | - | - |
| | Entreposé | 19-6-06 | 18h47 | MM Dans R12 |
| T045A | Récupéré | 21-06-06 | 8h40 | GPC P. sel. |
| | Entreposé | - | - | - |
| 06-07633A | Récupéré | 28-06-06 | 8:40 | FB 1x1.5ml P400 |
| | Entreposé | 28-06-06 | 9h10 | FB |
| 06-07634A | Récupéré | 28-06-06 | 9h00 | FB 1x1.5ml P400 |
| | Entreposé | 28-06-06 | 9h10 | FB |
| 06-07641 | Récupéré | 29/06/06 | 8:14 | SN MEX. P4 |
| | Entreposé | 29/06/06 | 11:03 | SN |
| 06-07626 | Récupéré | 29-6-06 | 9h20 | MM Prél. ~0.5ml pour reprise |
| | Entreposé | 29-6-06 | 10h22 | MM en triplicata de P600 |

Manipulations supplémentaires

(Suite)

| Code labo | Date | Heure | Initiales | Motif | |
|-----------------|----------|----------|-----------|-------|---|
| 06-07626 | Récupéré | 27/06 | 14h15 | MM | Prel. n°0, Smk pour C600 |
| | Entposé | 27/06 | 14h48 | MM | |
| 06-07626 A+B | Récupéré | 4/7/06 | 15h10 | CB | Transfert au congélateur |
| | Entposé | 4/7/06 | 15h13 | CB | |
| 06-07626 A+B | Récupéré | 11/09/06 | 10h07 | CB | Quintessence du B Prel. n°1 ml de chacune |
| | Entposé | 11/09/06 | 10h22 | CB | |
| CB | Récupéré | 2/ | 2/ | CB | Transférer aliquotes à S. Beau- leur 10h21 |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | Entposé | | | | |
| | Récupéré | | | | |
| | | | | | |

CHAIN OF CUSTODY DOCUMENTATION

Circle: BOTTLE OR ALIQOT(S) A OR B

Aliquot: SCREEN (BATCH OR INDIVIDUAL) OR INDIVIDUAL CONFIRMATION

Organization: _____ UCLA folder no. _____

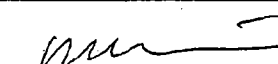
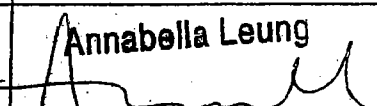
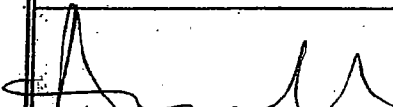
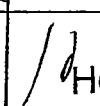
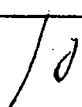
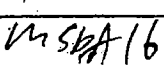
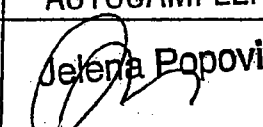
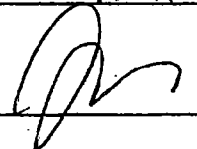

UCLA code numbers: NA

If individual conf., bottle no. _____ =UCLA code no. _____

If multiple replicate aliquots indicate here N = 2

Circle assay: I II CF TE FF EMIT EPO HCG DIU ONTRACK OTHER
(Pentane / Ether) (THC) (BZE) (MORPH) (AMPH)

IT IS REQUIRED TO PRINT EACH NAME AT LEAST ONCE ON THIS PAGE

| Released by: | To: | Date | Purpose |
|--|---|-------------|--|
|  Fereshteh Delshad | REFRIGERATOR #12 | MAY 31 2005 | STORAGE |
| REFRIGERATOR #12 |  Annabella Leung | MAY 31 2005 | Perform Assay |
|  | NAS Temp Storage | MAY 31 2005 | Temporary Storage |
| NAS Temp Storage |  Heith Williams | MAY 31 2005 | TRANSFER |
|  |  AUTOSAMPLER | MAY 31 2005 | Analysis, Then Storage |
| AUTOSAMPLER MSA 16 |  Jelena Popovic | JUN 01 2005 | Transfer |
|  | SAMPLE STORAGE SHELF | JUN 01 2005 | STORAGE  |
| | | | |
| | | | |
| | | | |

If batched with another group: _____

GDC0032

UCLA code: _____ and folder no. _____ (Int./date)

G:\SOP\LOGS&FRM\CUSTODY

Form by approved  1/13/05

CHAIN OF CUSTODY DOCUMENTATION

Circle: BOTTLE OR ALIQUOT(S)

A OR B

Aliquot: SCREEN (BATCH OR INDIVIDUAL) OR INDIVIDUAL CONFIRMATION

Organization: _____ UCLA folder no. _____



UCLA code numbers: NA

If individual conf., bottle no. _____ =UCLA code no. _____

If multiple replicate aliquots indicate here N = _____

Circle assay: I II CF TE FF EMIT EPO HCG DIU ONTRACK OTHER
(Pentane / Ether) (THC) (BZE) (MORPH) (AMPH)

IT IS REQUIRED TO PRINT EACH NAME AT LEAST ONCE ON THIS PAGE

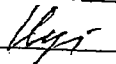
| Released by: | To: | Date | Purpose |
|---|--|-------------|-----------------------|
| FREEZER #15 |  Fereshteh Delshad | MAY 31 2005 | Aliquot Confirmation |
|  | Positive Freezer #2 | MAY 31 2005 | STORAGE JP 5/31/05 |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

If batched with another group:

UCLA code: _____ and folder no. _____ (Int./date) _____

GDC0033

G:\SOP\LOGS&FRM\CUSTODY

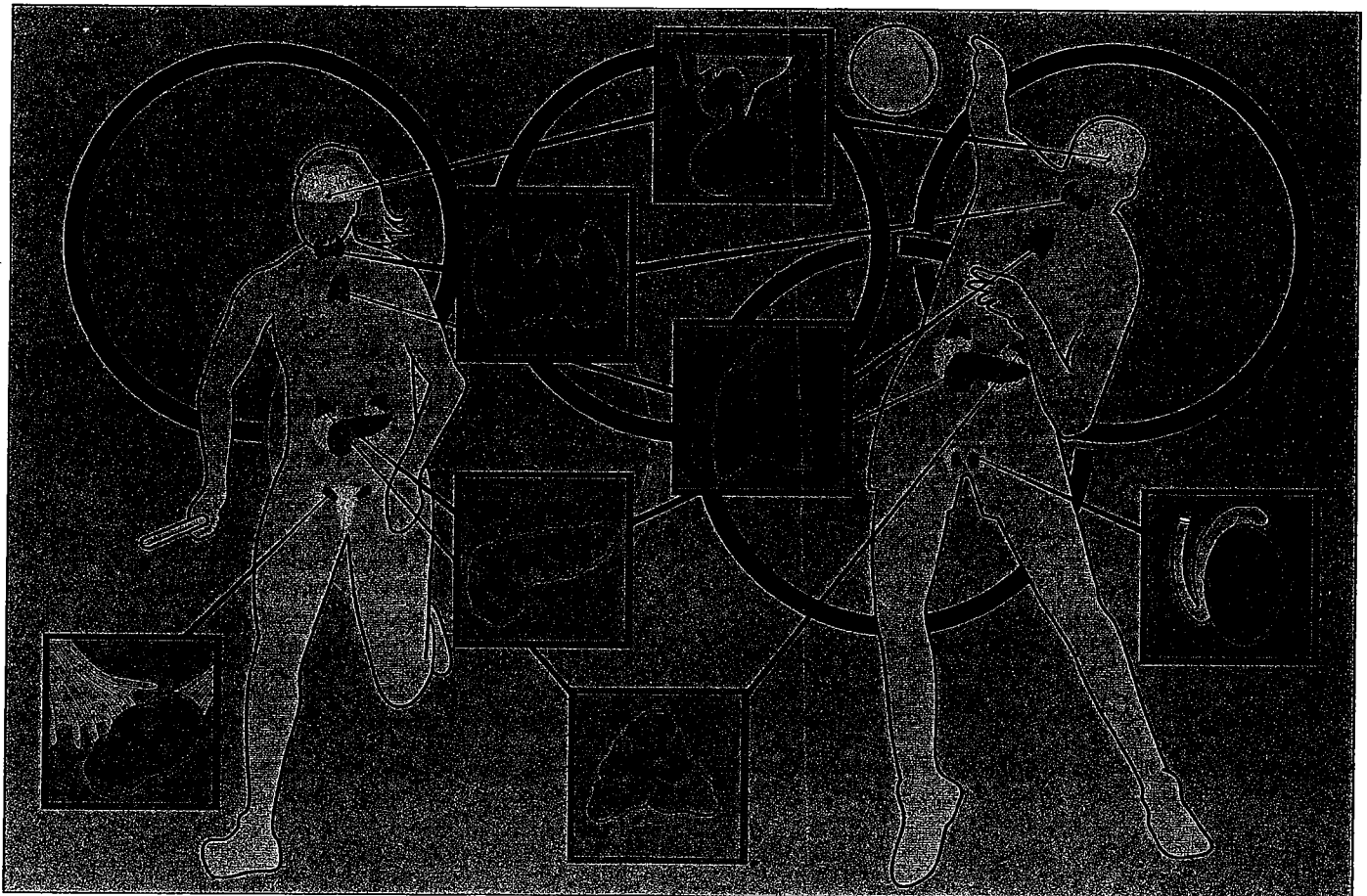
Form by approved  1/13/05

THE ENDOCRINE SYSTEM IN SPORTS AND EXERCISE

EDITED BY W.J. KRAEMER & A.D. ROGOL



THE ENCYCLOPAEDIA OF SPORTS MEDICINE
AN IOC MEDICAL COMMISSION PUBLICATION
IN COLLABORATION WITH THE
INTERNATIONAL FEDERATION OF SPORTS MEDICINE



Chapter 35

Effects of Testosterone and Related Androgens on Athletic Performance in Men

KARL E. FRIEDL

In spite of quantitative differences in the gene products of tissues from males and females, there are few important sexual differences in the genome, with the exception of the male-specific Y chromosome. The major function of this chromosome is to determine sexual differentiation; in its absence, ovaries develop. It is, then, the secretory products of the gonads that are responsible for sexual dimorphism. . . . As a consequence of its widespread action, testosterone is the major hormone responsible for the sexual dimorphism of nonreproductive tissues.

Bardin & Catterall 1981, p. 1285

Introduction

Although gender differences in body size and upper body strength may have been evident for centuries, the basis of these differences is still poorly understood. This ignorance hampers our ability to predict the effects of supplemental androgens administered to normal adult males for performance enhancement. Androgens administered to androgen-deficient men, prepubertal boys and normal women can produce marked effects on size and strength (Stanhope *et al.* 1988; Bhasin *et al.* 1997; Elbers *et al.* 1999), but it does not necessarily follow that an excess of androgens to normal men will provide more of the same effects along the same continuum. There is no specific disease of androgen excess in males, perhaps suggesting that more androgen does indeed produce more of the same biological effects (Can a man *be* over-virilized?) (Bardin *et al.* 1990). Alternatively, it could be reasonably expected

that limiting mechanisms simply will not allow open-ended or cumulative effects to occur (e.g. changes in receptor density and binding activity, negative feedback loops and non-linear interactions with other hormones) (Glass & Vigersky 1980). In this case, observed effects from supraphysiological doses of androgens may contrast those that occur in nature, producing pharmacological effects that are very different than those associated with male characteristics (e.g. stroke risk, gynecomastia, osmoregulatory changes). For practical purposes, supraphysiological could be defined as a dose that increases circulating levels of testosterone above the normal range, or other androgen formulations with rough bioequivalence to these above-normal testosterone levels (even though measured testosterone may be markedly suppressed); in some regards, any administration of exogenous androgen is supraphysiological because it does not precisely mimic circadian fluctuations and other properties of the body's own hormonal secretions. In the absence of data, speculative and anecdotal self-experiments by athletes have led to widespread gym lore about androgen use, and non-users are left with concerns about unfair performance advantages. A greater understanding of androgen physiology can improve the scientific basis of training regimens for athletes and help define the full range of modifiable human performance. This chapter considers the available data on testosterone and related androgenic steroid effects on athletic performance and attempts to expand on earlier thoughtful reviews (Wright 1980; Haupt & Rovere 1984; Wilson 1988; Yesalis 2000).

Metabolism and action of testosterone and related androgenic steroids

The basic physiology of testosterone production and secretion is relatively well known, while the far-reaching effects of testosterone on cells throughout the body, as well as interactions with other anabolic hormones, are not as well understood (Bardin & Catterall 1981). Testosterone is a steroid hormone that is made from cholesterol through a series of enzyme-regulated steps in the Leydig cells of the testes of men, and in the adrenal cortex of men and women. The key regulator of the testicular secretion is luteinizing hormone (LH), a protein hormone from the pituitary gland. LH secretion, in turn, is regulated by gonadotropin-releasing hormone (GnRH), which is regulated in the hypothalamus. LH and GnRH secretion are controlled, in part, by circulating levels of testosterone in a negative feedback loop. If testosterone or a related androgenic compound is administered artificially, the system down-regulates, reducing the amount of testosterone synthesized by the testes. If higher than normal circulating levels are sustained by administration of androgen, the testes substantially reduce testosterone and sperm production, reducing the volume of the testes as the seminiferous tubules are depopulated of maturing germ cells (a readily reversible phenomenon). This noticeable reduction in testicular volume (Kiraly 1988; Friedl *et al.* 1991) is one of the androgen effects that make blinded studies in androgen-experienced athletes difficult to conduct.

Testosterone's effects on body tissues are far more complicated than its production and secretion. Testosterone is directly active in most tissues, acting through a specific androgen receptor. Additionally, in some tissues such as the prostate and hair follicles, testosterone is converted to 5 α -dihydrotestosterone (DHT). DHT has greater potency than testosterone on androgen receptors, but is also more rapidly metabolized to biologically inactive 5 α -androstane diols. In fat cells, where high concentrations of aromatase enzyme are present, some of the testosterone is converted to estrogen, with estrogen actually playing important roles in the mediation of apparent testosterone actions in some tissues such

as intra-abdominal fat metabolism, regulation of key hepatic proteins, bone mineral metabolism and some brain effects (e.g. regulation of gonadotropin secretion). In muscle cells, testosterone appears to act directly on androgen receptors, which are present in much lower density than in more androgen-responsive tissues such as prostate, and then is excreted as a water-soluble conjugate, 3 α -androstane diol glucuronide; muscle lacks the 5 α -reductase to convert testosterone to DHT (Michel & Baulieu 1980; Hughes & Krieg 1988). Androgens, including testosterone and DHT, are bound to a variety of carrier proteins in circulation, most specifically to sex hormone binding globulin (SHBG), but also non-specifically to albumin. This constitutes a large circulating 'reserve' pool of immediately available steroids that has some protection against metabolism and excretion while bound, acting in a kinetic balance with receptors and competitive ligands that may vary between tissues, with only 1–2% unbound (or 'free') at any time. Testosterone activates the secretion of other potent anabolic hormones such as insulin-like growth factor I (IGF-I) and erythropoietin, and potential benefits to athletic performance may accrue from these indirect effects of androgen. Localized effects of testosterone, including such interactions at the tissue levels, may be very important and are just beginning to be appreciated with the availability of modern research methods such as transgenic animal studies. Testosterone can also bind to other receptors such as glucocorticoids, in this case providing competitive inhibition of certain catabolic stress responses induced by cortisol; thus, part of the anabolic action of testosterone may actually be exerted through this crossover binding to modify catabolic responses (Hickson *et al.* 1984; Janne 1990).

Pharmacological compounds that have been developed to extend or modify the effect of testosterone build on some of these understood effects of androgens (Liddle & Burke 1960; Kruskemper 1968; Kochakian 1988). Thus, steroids that cannot be converted to estrogenic compounds or to DHT will exhibit characteristic effects different from those of testosterone, with reduced effects on bone or prostate, respectively (Sundaram *et al.* 1995). Some steroid-using athletes include aromatase inhibitors

in the drug regimen to block conversion to estrogens, or use estrogen receptor blockers to prevent estrogenic actions, in attempts to prevent gynecomastia and other side effects (Friedl & Yesalis 1989). Most androgenic-anabolic steroids have been developed to reduce the 'androgenic' effects on reproductive tissues such as prostate, in favor of 'anabolic' effects on muscle and bone; these would focus on steroid structures that cannot be readily reduced to DHT (Hershberger *et al.* 1953). Testosterone administered either orally or by injection promptly reaches circulation and is rapidly removed by the liver and metabolized to products that can be readily excreted in urine or bile. Modifications with 17-alkylation protect testosterone against this rapid elimination, making methyl testosterone an orally active androgen, but the metabolites produced in various tissues are 17-alkylated compounds that will exhibit different binding properties and biological effects. Adverse effects on the liver are one of the properties inherent in the orally active 17-alkylated androgens. Methandienone (Dianabol) is the most important orally active 17-alkylated androgen in the studies of athletic performance (Friedl 2000). This was the orally active drug of choice by steroid-using athletes in the 1960s and 1970s but has been since taken off the market by major drug manufacturers (Yesalis *et al.* 1988). Modifications of testosterone with an ester side chain also protects against rapid elimination, but because this side chain can be hydrolyzed to provide graded release of testosterone into circulation from deep intramuscular injection sites, the effects are different than those of the 17-alkylated compounds. The duration of action of these esterified androgens is roughly dependent on the length of the side chain; a relatively short side chain in testosterone undecanoate makes this a relatively short acting testosterone (usually used orally) (Schurmeyer *et al.* 1983), while high levels of testosterone can be maintained with testosterone enanthate (TE) or testosterone cypionate in weekly injections, and the very long chained testosterone buciclate maintains circulating levels for months (Behre & Nieschlag 1992). A compound that differs from testosterone only by the elimination of one methyl group at the 19 carbon position ('19-nortestosterone' or nandrolone) was

developed to try to reduce androgenic side effects on prostate through its properties that prevent aromatization to estrogens and its metabolism to compounds that are weaker than DHT (e.g. 19-nordihydrotestosterone) (Hershberger *et al.* 1953; Sundaram *et al.* 1995). Nandrolone esters (e.g. nandrolone decanoate or 'Deca-Durabolin') and testosterone esters (e.g. TE) are the most commonly used androgenic preparations today, and these are the compounds that have been used in the majority of modern studies on performance effects. A wide variety of androgens are active in muscle, while requiring greater selectivity for some other tissues; for example, protection of bone against glucocorticoid effects requires an aromatizable androgen, while nandrolone compounds are effective in preserving muscle but not bone (Crawford *et al.* 2003). Any administration of exogenous steroid is non-physiological, even at doses that restore mean circulating levels to 'normal', as there is a large fluctuation in normal circulating levels of testosterone during the day.

Dietary supplements that are legally marketed as 'steroid replacers' in the USA include a wide range of products (e.g. sterols, sapogenins, androgen metabolites, yohimbe bark, boron), none of which have any convincing scientific basis for performance enhancement in normal men. Some products have been tainted with banned substances that will give a positive drug test; others are simply bogus and even harmful (Friedl *et al.* 1992b). There is no over-the-counter drug that comes close to the actions of testosterone, or significantly stimulates its production or release in the body (King *et al.* 1999). If such an effective product surfaced, it would likely be added to the list of androgens and other substances banned by athletic federations.

Most recently, new 'designer' steroids have been detected in urinalyses from elite athletes (Catlin *et al.* 2002). Tetrahydrogestrinone is a compound related to gestrinone, a steroid used to treat endometriosis in women, and is the latest to turn up in drug testing in a wide variety of sports including track and field, football and boxing (Knight 2003). The novelty is not in some new marvelous mechanism of action, but rather in the fact that the compound was new to human use and therefore not

specifically tested for in routine drug screens. Other synthetic steroids have been administered in attempts to correct the abnormal profile of androgen metabolites that signal exogenous use of androgens (Aguilera *et al.* 2002). The use of new drugs with little or no evaluation of human safety should be of great concern to normal healthy athletes.

For purposes of this chapter, anabolic steroids are used to denote synthetic androgenic steroids other than the testosterone compounds, but are used somewhat interchangeably, as anabolic and androgenic properties are notional and have never been effectively separated (Kochakian 1988).

Is there a male athlete hypogonadal equivalent to athletic amenorrhea?

Environmental regulators of testosterone secretion

Female athletes have been commonly warned that reproductive cycles may cease with too much exercise, as part of a 'syndrome' involving exercise, amenorrhea and bone mineral loss. Extrapolations from the theory of an athletic amenorrhea in men suggest that with high volume physical training, testosterone will be suppressed in male athletes. Furthermore, it has been suggested that these athletes might benefit from 'replacement' androgen therapy. This interpretation may have started with military training studies in which marked reductions in testosterone were noted in healthy young men (Aakvaag *et al.* 1978). In normal men working hard with inadequate energy intake, testosterone can fall nearly to castrate levels. This has been shown in high school wrestlers during their wrestling season while they are energy-restricted (Strauss *et al.* 1985; Roemmich & Sinning 1997) and in elite soldiers during short- and long-term intensive training (Opstad 1992a; Friedl *et al.* 2000). Guezennec *et al.* (1994) demonstrated a testosterone decline with increasing energy deficit in French commandos. However, testosterone levels are rapidly restored to normal with refeeding, even in the face of other persistent stressors such as workloads exceeding 25 MJ·day⁻¹ (6000 kcal·day⁻¹) (Friedl *et al.* 2001). Thus, these large reductions, generally much

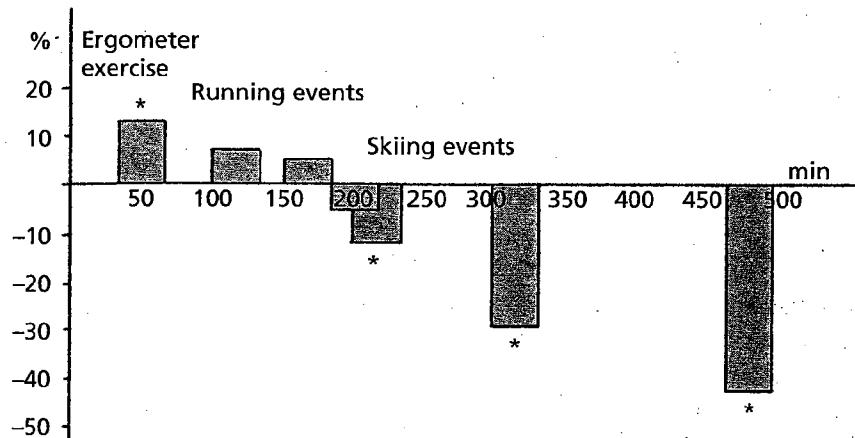
larger than those observed in athletes in training, can be explained by inadequate energy intake and not as an exercise-specific effect (Friedl 1997). As it turns out, even the concept of a female athletic amenorrhea produced by high volume exercise may not be correct; earlier studies were confounded by under-reporting food intake. As observed in men, energy deficit, not exercise, appears to be the key stressor affecting reproductive cycles in women (Loucks & Thuma 2003; Loucks 2004).

Psychological factors also affect testosterone levels in healthy men. The thrill of victory may produce short-term increases in circulating testosterone levels (Ursine *et al.* 1978; Elias 1981), while anxiety produces reliable declines (Bourne *et al.* 1968; Kreuz *et al.* 1972; Francis 1981). Mood and motivation in human research is difficult to control or ethically manipulate. However, in one novel study of men in parachute training making a stressful jump out of a high tower, the initial jumps resulted in a marked stress effect with acute suppression of testosterone and elevation of cortisol ('distress'), while later experienced jumps led to acute elevation of testosterone and no change in cortisol ('eustress') (Ursine *et al.* 1978). There is a large body of literature on the effects of defeat and chronic stress on testosterone suppression in animals that suggests this is a key regulator of normal testosterone in mammalian species (Blanchard *et al.* 1993). In humans, chronic anxiety appears to have a prominent suppressive effect on testosterone and may even provide a more reliable indicator of a noxious stress than traditional measures of the hypothalamic-pituitary-adrenal axis such as cortisol (Kreuz *et al.* 1972; Francis 1981; Hellhammer *et al.* 1985).

Effects of resistance and endurance exercise on testosterone

Exercise itself does not appear to be an important regulator of testosterone secretion through either primary (actions at the testes) or higher levels (actions at the pituitary or hypothalamus), although little is known about the neurobiology of exercise (Rogol *et al.* 1984), or any direct signals from exercising muscle that may affect the gonadal axis. Endurance athletes have been variously reported

Fig. 35.1 Apparent relationship between duration of exercise and serum testosterone from various studies, suggesting a role for a progressive metabolic stimulus such as declining glucose availability on regulation of testosterone secretion. (From Viru 1985.)



to have modest reductions in resting testosterone levels (Wheeler *et al.* 1984; De Souza *et al.* 1994), or no differences (Bagatell & Bremner 1990; Lucia *et al.* 1996), compared to sedentary controls. Male runners reducing their weekly mileage from 81 km·wk⁻¹ to 24 km·wk⁻¹ demonstrated no change in low, normal testosterone levels, even though creatine kinase levels were reduced by half, reflecting a reduction in muscular strain (Houmard *et al.* 1994). The biological significance of this altered status to low-normal circulating levels observed in some studies is unknown.

Exercise can acutely increase or decrease circulating testosterone, depending on the mode and intensity of exercise (Schmid *et al.* 1982; Viru 1985). Increases occur during and after relatively short, high intensity work such as resistance training or sprint events, while declines are associated with increasing duration endurance events and are especially noted in marathon and ultra-running events. Endurance exercise has been reported to decrease testosterone by as much as half, even after appropriate comparison to same time-of-day baseline values (Dessypris *et al.* 1976; Morville *et al.* 1979; Kuoppasalmi *et al.* 1980; Schurmeyer *et al.* 1984). These effects observed during or shortly after the exercise period, may persist into the next day or two after cessation of exercise. The physiological consequences of this short-term suppression are uncertain; this decline is comparable to the changes observed daily in normal men, and the observed reductions still do not fall into the range of a clinical

hypogonadism that would normally justify treatment. Mechanisms for this change with prolonged exercise have been variously postulated and investigated. Catecholamine effects on testicular blood flow (Collu *et al.* 1984) and cortisol suppression of testosterone production (Bambino & Hsueh 1981) do not seem to occur at physiological levels (Sapolsky 1985, 1986). Increased clearance rates of testosterone by exercising muscle (e.g. increased excretion of 3 α -androstenedione-glucuronide) appears to occur (Ponjee *et al.* 1994), but could be construed as an increased biological action reflected in greater turnover at the muscle target site, rather than a deficiency state. It may be that this transient decline in testosterone that appears to increase with duration of exercise (Fig. 35.1) is explained simply by a within-exercise energy deficit, highlighting the sensitivity of GnRH to low glucose levels in the hypothalamus (Opstad 1992b; Loucks 2004). A similar effect of a progressive 'starvation' profile has been suggested for changes in thyroid hormone levels during prolonged exercise (O'Connell *et al.* 1979). Some extreme endurance athletes recognize the need for liquid carbohydrate replenishment during their exercise (Saris *et al.* 1989), although whether or not this better sustains testosterone levels and muscle mass has not been studied.

In contrast to prolonged endurance exercise, resistance and sprint exercise usually produces short-term elevation in testosterone concentration (Sutton *et al.* 1973; Cumming *et al.* 1986; Kraemer *et al.* 1991), or no change observed (Guezennec *et al.*

1986). Several mechanisms would be reasonably expected to temporarily increase serum concentrations. Liver blood flow markedly declines with increasing intensity of exercise (Rowell *et al.* 1964) and would reduce hepatic clearance and temporarily increase serum concentrations. Fluid shifts with movement out of the vascular space during exercise (Wilkerson *et al.* 1980) would increase the concentration of all protein-bound hormones such as testosterone, which cannot move with the fluid. Dopamine and prolactin may regulate testosterone release, but in one study, testosterone changes were unaffected when the exercise-induced rise in prolactin was blocked by L-dopa (Jezova-Repceckova *et al.* 1982). Sympathetic activity may also play a role in exercise-induced testosterone increase (Jezova & Vigas 1981).

Effects of endogenous testosterone changes on performance

Temporary exercise-induced declines in testosterone may not have much effect on body composition, as much more profound reductions in testosterone lasting weeks have relatively small effects. Artificial hypogonadism induced for 10 weeks in healthy young men who were not exercising produced a 2 kg shift in lean-to-fat tissue balance (Mauras *et al.* 1998). In healthy young soldiers who were underfed while working hard, individual differences in the level of testosterone suppression was a relatively small contributory factor in the proportion of lean tissue lost during weight loss over 8 weeks, even though the association was statistically significant (Friedl 1997). This is supported by findings in animal studies, where fasting and exercise caused smaller testosterone reductions in endurance-trained animals and this difference was also associated with higher lipolysis and decreased glycogenolysis, possibly protecting muscle protein (Guezennec *et al.* 1984b).

Acute increases in testosterone observed with resistance exercise may be enough to trigger tissue receptor-level changes or other anabolic hormones, but this remains unknown. In training studies conducted by Kraemer *et al.* (1991), a comparison of bodybuilder to power lifter routines with differ-

ences in repetitions and duration of rest periods between sets demonstrated large differences in growth hormone responses, but a minimal difference in the magnitude of testosterone increases in either routine. One can only speculate on the significance of brief testosterone changes in either direction to synergistic actions with other musculotrophic hormones such as local tissue activity of IGF-I, including in the final determination of muscle hypertrophy under the influence of strength demands for strength athletes and the slimming down of muscle mass that would be advantageous for distance runners.

Effects on muscle mass: how big can humans get?

Increases in total body weight

Androgen supplementation to normal men increases body weight and amino acid incorporation into muscle protein. The precise nature of the observed weight gain, how much can be added with continuous androgen administration, and how much will remain after steroid cessation is far from clear. In general, humans increase or lose lean and fat mass in roughly the same proportions as they move up and down the scale of energy balance, with approximately two-thirds of the changes in fat mass. Forbes has plotted these trends on the basis of data from overfeeding and underfeeding studies (Fig. 35.2) (Forbes 1993). He suggests that supplementation with anabolic hormones moves individuals off the normal trajectory, with a substantial increase in the lean component and even a reduction in the fat component. In other words, androgen supplementation has been suggested to provide a nutrient partitioning effect similar to the class of β -adrenergic agonist drugs that have been developed for animal food production (e.g. clenbuterol) that favor a specific increase in the lean mass component. Forbes was one of the first to illustrate this principle in a study with high-dose androgen administration to normal men (see Fig. 35.3, below) (Forbes *et al.* 1992). However, all studies to-date, including this one, leave nagging questions about the role of dietary intakes, training effects, and the

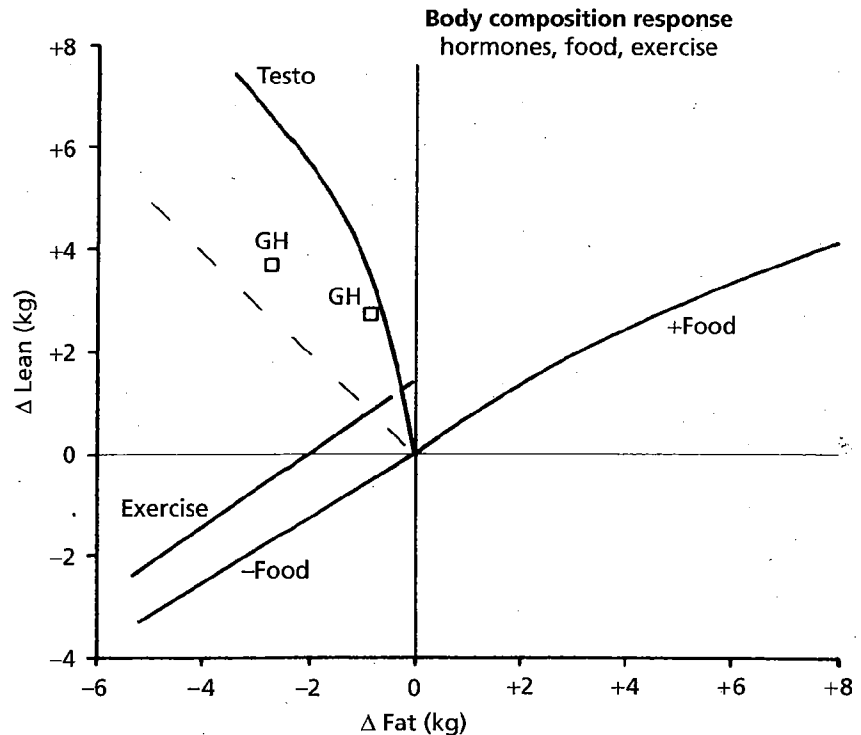


Fig. 35.2 Conceptual relationship between fat and lean components during changes in body weight and the effect of superimposed anabolic hormone administration. GH, growth hormone. (From Forbes 1993.)

validity of the assumptions underlying each of the methods used to estimate these changes.

Weight gain is a well-known side effect of androgens used in clinical practice and in male contraception trials using supraphysiological doses of injectable testosterone esters (typically 200 mg·wk⁻¹) (World Health Organization Task Force 1990; Young *et al.* 1993). A typical period of 6 weeks or longer of continuous testosterone ester administration to normal adult males provides about 3–5 kg of weight gain within the first few weeks (Mauss *et al.* 1975; Friedl *et al.* 1990, 1991; Welle *et al.* 1992; Young *et al.* 1993; Pope *et al.* 2000). Orally active androgens have the same effects. A large series of studies in the early 1970s with methandione (Dianabol) showed consistent increases of about 2 kg in men given at least 10 mg·day⁻¹ for 3–4 week periods, regardless of physical training status (Friedl 2000). It is not clear that this weight gain effect is either dose- or time-dependent. In the male contraceptive studies that have given androgen (testosterone enanthate, TE) at a dose of 200 mg·wk⁻¹ for more than a year, the individuals do not continue to gain weight, despite the sustained doubling or tripling of circulating testosterone levels with this

dosing regimen. There were no effects in normal men at typical doses used in replacement therapy for hypogonadal men (e.g. 100 mg·wk⁻¹ of testosterone cypionate or nandrolone decanoate, ND) (Crist *et al.* 1983), but doubling the dose (ND, 200 mg·wk⁻¹ for 8 weeks) produced significant increases in weight (Hartgens *et al.* 2001). In another study, androgen doses that produce 'replacement' levels in normal men (TE, 100 mg·wk⁻¹) did not change body weight, while high levels of TE (300 mg·wk⁻¹) and two doses of ND (100 mg·wk⁻¹ and 300 mg·wk⁻¹) produced significant weight gain (Friedl *et al.* 1991). A study that was twice as long with comparable weekly dosing (TE 280 mg·wk⁻¹) did not cause twice as much weight gain (Friedl *et al.* 1990). Bhasin *et al.* (1996) doubled the weekly dose of testosterone (TE, 600 mg·wk⁻¹ for 10 weeks) and also observed a similar final increase of about 3.5 kg, while in the group of men who included exercise with the same high dose, this weight increase nearly doubled. Pope *et al.* (2000) found an increase of 3.0 kg following a typical bodybuilder regimen of increasing doses of testosterone cypionate from 150 mg·wk⁻¹ up to 600 mg·wk⁻¹ over 6 weeks. (They reported all of this weight gain as

lean mass on the basis of skinfold thicknesses that showed little change with androgen.) Although Forbes has suggested a dose-response relationship to lifetime exposure to androgens (Forbes 1985), these data suggest that supraphysiological doses of androgens provoke a specific weight increase as a threshold effect, after which mechanisms adjust and prevent further gains. If this were not true, one would expect all normal men to continue to gain weight from cumulative androgen exposure after the initial pubertal surge in testosterone, instead of reaching stable weights in the early 20s without further dramatic gains.

These increases in weight observed in controlled studies and clinical observations of high-dose androgen administration to normal men reflect only a portion of the apparent weight differences that have been estimated for anabolic steroid-using bodybuilders. In dedicated bodybuilders, Kouri *et al.* (1995b) found a difference in weight between steroids users and non-users of nearly 10 kg, with comparable estimates of adiposity between the groups (~12.5% body fat). A comparison of the data for 20 presteroid era Mr. America winners (1939–1959) to a group of popular modern bodybuilders indicated an estimated difference of 20 kg of mass, presumably lean mass. Changes in nutrition and health care over the past century have increased the lean mass of typical young US soldiers by nearly 10 kg (Friedl 2004). Thus, it would be reasonable to ascribe some of the weight gain in bodybuilders since the 1940s and 1950s to a combination of this improved nutrition, as well as exercise science techniques; however, Kouri's data provide at least a rough estimate of the upper limit of the mean gain that might be attributable to androgen use. The difference of 3–5 kg gains in controlled trials and 10 kg seen in androgen-using bodybuilders may reflect an effect of repeated cycles of use, as well as training and dietary interactions.

Characterizing changes in body composition

The nature of androgen-induced weight gain has never been adequately characterized. A key problem is that most studies have measured body composition using only one technique, with each

technique dependent on certain assumptions that may not hold true with anabolic steroid administration. Although the combined error of multiple techniques in a multicompartiment model of body composition is generally smaller than the biological variation in body compartments measured (Friedl *et al.* 1992a), multicompartiment models may also not resolve the question if steroid-induced changes confound the assumptions of these methods. For example, if a portion of the androgen response includes an increase in total body water relative to the total lean mass, as has been suggested but never properly tested (Freed *et al.* 1975; Hervey *et al.* 1976; Wilson 1988), this could produce significant errors in the estimated changes determined by each of the principal body composition techniques employed, by violating the assumptions about the composition of the lean compartment for each method. Underwater weighing assumes 1.1 g·cm⁻³ density of lean tissue, dual energy X-ray absorptiometry (DEA) assumes an attenuation coefficient based on normally hydrated soft tissue, potassium-40 depends on a fixed distribution in the lean compartment (66.6 mmol·kg⁻¹), and deuterium dilution assumes a fixed hydration level (usually 72%) of the lean mass. These might not be trivial errors. In semi-starvation of normal men, where hydration of the lean mass increases in a subclinical edema, DEA measures 3.5 kg more tissue than is actually present, based on simple scale weights (Friedl *et al.* 1994). Although speculative, a change in the opposite direction with rapid increase in body mass produced by androgens could also be accompanied by increased water retention. If this is the case, each of the body composition methods would be expected to incorrectly estimate the changes in the lean mass component of weight.

Hervey attempted to resolve the question in his studies of high-dose methandienone (100 mg·day⁻¹ for 6 weeks). Total body potassium measurements predicted that an average weight increase of 3.3 kg was composed of 6.3 kg lean mass gain and 2.5 kg fat loss. However, body density measured by underwater weighing predicted 2.4 kg lean mass gain and 0.9 kg fat gain, and skinfold thicknesses did not change (Hervey 1976). A second study with the same dosing produced comparable average

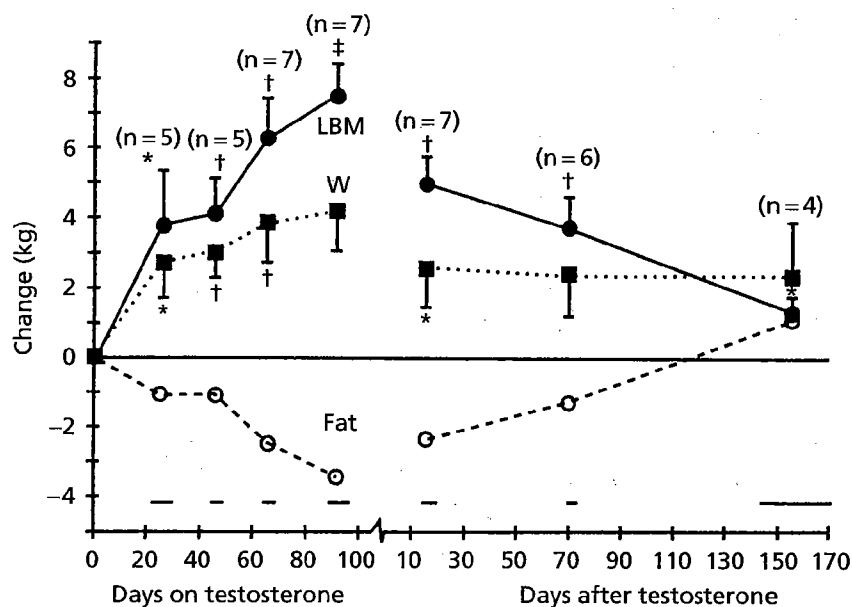


Fig. 35.3 Changes in body weight and lean and fat mass components in healthy young men administered $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{wk}^{-1}$ testosterone enanthate for 12 weeks and following cessation of drug administration. LBM, lean body mass; W, weight. (From Forbes *et al.* 1992.)

weight gains of 3.6 kg and similar changes in the total body potassium (Hervey *et al.* 1981). This time, total body nitrogen was also measured by neutron activation analysis, with an estimate that if the increased body weight reflected new muscle, body nitrogen should increase by about 100 g. The measured increase was more than double this value, raising new questions about the nature of the increase.

Forbes *et al.* (1992) reported on a typical regimen of approximately $200 \text{ mg} \cdot \text{wk}^{-1}$ of TE for 12 weeks, showing an average weight gain of 4.1 kg (Fig. 35.3). Based on potassium-40 measurements, he estimated an increase in the lean mass component of 7.5 kg by the end of 12 weeks of drug administration. This was corroborated by urinary creatinine excretion that increased by an average $358 \text{ mg} \cdot \text{day}^{-1}$ (on a meat-free diet), corresponding to an estimated lean mass increase of 8.6 kg.

There is an apparent rapid loss from the lean mass compartment following androgen cessation. In this same study, Forbes *et al.* (1992) reported a prompt decline in weight to about 2.5 kg within the first 10 days following steroid cessation, and this new weight was maintained over the next 6 months (Fig. 35.3). The estimated lean mass declined to about half of the peak gain by 2 months following steroid cessation, and further declined over 6 months to just over 1 kg from the initial baseline.

This was balanced at stable weight by an assumed regain of fat weight following an initial average loss of 3.4 kg. The loss of at least some of the weight gain following steroid cessation is comparable to the observations in normal men in whom testosterone is artificially suppressed. Normal men artificially reduced to prepubertal levels of circulating testosterone for 10 weeks had a 2 kg loss of lean mass, with a comparable increase in fat mass, as measured by DEA (Mauras *et al.* 1998). This suggests a labile component of lean mass that has some dependence on threshold changes in androgen levels.

Several studies have used imaging or muscle biopsies to conduct morphological analyses on the cross-sectional area of muscle and muscle fibers. Histological analyses of muscle biopsies from the vastus lateralis of normal subjects following administration of TE $200 \text{ mg} \cdot \text{wk}^{-1}$ for 6 weeks was not able to demonstrate significant increases in muscle fiber diameter (Griggs *et al.* 1989), while two other studies using athletes found significant increases in muscle fiber area of this muscle (Alen *et al.* 1984; Kuipers *et al.* 1993). A more recent study found that TE 300 and $600 \text{ mg} \cdot \text{wk}^{-1}$ for 20 weeks in normal men with endogenous androgen suppression significantly increased the cross-sectional area of muscle fibers and myonuclear number in biopsies of the vastus lateralis, indicating an effect of androgen on muscle

hypertrophy; there was no significant change in the proportion of type I and type II fibers (Sinha-Hikim *et al.* 2002). This same study demonstrated dose-dependent increases in vastus muscle volume measured by magnetic resonance imaging (MRI), and an apparent dose-dependent increase in total lean mass at 300 and 600 mg·wk⁻¹ TE. In their earlier study, Bhasin *et al.* (1996) found significant increases in MRI-measured muscle cross-sections of both the triceps and quadriceps of the men receiving testosterone, whether or not they included exercise. However, no change was found for the exercise alone group, which supports the overall effect of androgens on muscle mass increase. New state-of-the-art techniques to quantify skeletal muscle should be of great value in terms of ease and precision for future studies (Gallagher *et al.* 1999).

Androgens increase muscle protein synthesis, but do not typically cause an increase in total body protein synthesis. Griggs *et al.* (1989) studied healthy men administered approximately 200 mg·wk⁻¹ TE for 12 wks and found typical increases in lean mass based on potassium-40 and creatinine excretion, accompanied by a 27% mean increase in muscle protein synthesis rates. The observed increases are therefore presumed to be a shift in the balance between synthesis and degradation rates, with an overall increase in turnover rates. Presumably, the effect is mediated directly through androgen receptors in the muscle, but this mechanism has not been well characterized, and there appear to be major differences in the androgen responsiveness of different muscles (Gustafsson *et al.* 1984; Hughes & Krieg 1988; Antonio *et al.* 1999). New studies of androgen-receptor signaling pathways in muscle are beginning to identify an inverse relationship between new muscle cell development and adipocyte development that appear to link androgen effects on fat and muscle shifts (Lee 2002), as has been previously suggested for steroid hormone actions in bone in the balance of osteoblasts and adipocytes. Other transgenic studies that use targeted overexpression of androgen receptor (Wiren *et al.* 2003) and muscle-specific IGF-I (Paul & Rosenthal 2002) in muscularly overdeveloped 'mighty mouse' animal models are likely to produce near-term breakthroughs in our understanding of bone, muscle and fat regulation by androgens,

as well as the essential role of estrogens in these effects.

Studies of regional fat tissue metabolism suggest that androgens reduce triglyceride accumulation in intra-abdominal fat, but do not have this effect on subcutaneous fat (Marin 1995; Marin *et al.* 1996), although testosterone treatment produces an increase in visceral fat in women (Elbers *et al.* 1997). An increase in lean mass might further influence body composition by changing energy requirements, but there is no apparent androgen-specific increase in energy metabolism. Welle *et al.* (1992) found a 7% increase in basal metabolic rate with TE (200 mg·wk⁻¹ for 12 weeks), and this increase could be completely accounted for by the increase in skeletal muscle mass. An informative study of the body composition changes was conducted using the 'Leydig clamp' model of Bhasin and his colleagues, with suppressed endogenous testosterone combined with sub- and supraphysiological doses of testosterone enanthate (25–600 mg·wk⁻¹ for 20 weeks) (Bhasin *et al.* 2001). Changes in lean men were assessed by DEA, including regional lean and fat estimates, and by multiple MRI slices of the thigh and abdomen (Woodhouse *et al.* 2004). The DEA data indicate increases in truncal and extremity lean mass with high doses, and increases in fat mass with subphysiological doses. MRI scans indicated increases in adipose tissue volumes in all regions assessed (intra- and subcutaneous abdominal, and intermuscular and subcutaneous thigh) at low doses, with less consistent reductions in adipose tissue volume at supraphysiological doses. These data provide confirmation of androgen-induced increases in lean components of body composition and decreases in fat mass.

Mechanisms of performance enhancement

Are observed strength gains only from increased muscle mass?

An increase in muscle size is associated with increases in strength, since maximal force production in a muscle is directly proportional to the cross-sectional muscle area. It has been suggested that androgens produce their main effects simply

through the increase in muscle size, without other changes in neuromuscular performance (Schroeder *et al.* 2003). There are other possible explanations for androgen-induced strength gains in athletes, including neuromuscular adaptations and muscle biochemical and physiological alterations, but these remain largely undefined. One of the first published strength studies in normal men involved four medical students given $50 \text{ mg} \cdot \text{day}^{-1}$ methyl testosterone along with creatine, and tested for changes in grip strength in a single-blind, crossover study with 3 weeks of steroid use (Samuels *et al.* 1942). No improvements in performance were observed. Among the difficulties in objectively assessing the direct effects of androgens on muscular strength are the variability inherent in strength tests, establishing appropriate controls for both individual experience and current training status, blinding the subjects to their treatment (because of obvious changes such as testicular volume), and the potentially important interactions with other factors that may also be influenced by androgens, such as motivation and training during the study.

Earlier studies with methandienone ($10 \text{ mg} \cdot \text{day}^{-1}$, or more, for 3–4 weeks) produced significant improvements of approximately 15% in one repetition maximum (1 RM) bench press performance, including some blinded tests and a mix of trained and untrained subjects, as previously reviewed (Haupt & Rovere 1984; Friedl 2000). Methandienone was quite consistent in increasing strength performance, although considerable discussion about the interactions with exercise has never been resolved (e.g. Freed *et al.* 1975; Hervey *et al.* 1981). With careful blinding, including a testosterone 'replacement' comparison dose group of TE ($100 \text{ mg} \cdot \text{day}^{-1}$), a high dose of TE ($300 \text{ mg} \cdot \text{day}^{-1}$ for 6 weeks) produced significant increases in elbow flexion and knee extension tests (Friedl *et al.* 1991). Despite equivalent gains in body weight in ND treatment groups 100 and $300 \text{ mg} \cdot \text{day}^{-1}$, there were no significant increases in strength. An increase in hip adduction was the only muscle strength measure that changed in untrained men receiving TE ($200 \text{ mg} \cdot \text{wk}^{-1}$ for 24 weeks) (Young *et al.* 1993). Bhasin *et al.* (1996) measured an increase in 1 RM bench press with TE ($600 \text{ mg} \cdot \text{wk}^{-1}$) and demonstrated that steroid and exercise each produced significant gains that were

additive in the steroid and exercise group (Fig. 35.4). In Bhasin's more recent dose-response study, TE (300 and $600 \text{ mg} \cdot \text{wk}^{-1}$) significantly increased 1 RM leg press (average increases of $\sim 75 \text{ kg}$) and leg power ($\sim 40\text{--}50 \text{ W}$ increase), while no changes were observed at replacement or lower doses (Bhasin *et al.* 2001). This corresponded to increases in thigh volume and muscle fiber size in these two groups. These strength changes did not appear to be dose-dependent. No changes were observed in leg press repetitions to failure ('fatigability') (Storer *et al.* 2003).

Separate from the effects of muscle cross-sectional area, effects on psychomotor enhancement have been suggested. Methandienone was reported to significantly shorten patellar reflex time in weight lifters (Ariel & Saville 1972), but an intensive study of a small group of elite steroid-using Finnish strength athletes showed no changes in psychomotor or motor speed tests compared to a control group of athletes (Era *et al.* 1988). Both groups improved physical performance during 24 weeks of training, without an apparent androgen effect (Alen *et al.* 1984).

Aggression and motivation

Testosterone has become synonymous with aggressive power. One of the most difficult aspects of studying androgen performance effects is ensuring that the subjects are blinded to the treatment, in part because many subjects perceive a change in their mental outlook when they are receiving high-dose androgens (Freed *et al.* 1975; Wright 1980). The change in mental status has been variously described as an increase in feelings of energy, a compulsive focus on winning their event, and complete disinhibition of aggressiveness, even to the point of murderous rage. Pope has reported extensively on the psychological effects of androgen supplementation for over a decade (e.g. Pope & Katz 1988, 1994). Pope's research team found significant neuropsychological effects in a thoughtful study designed to mimic the increase in androgen dose employed by many bodybuilders by stepping the weekly dose up from $150 \text{ mg} \cdot \text{wk}^{-1}$ to $300 \text{ mg} \cdot \text{wk}^{-1}$ to $600 \text{ mg} \cdot \text{wk}^{-1}$ (Kouri *et al.* 1995a; Pope *et al.* 2000). In this, and other studies reviewed by Pope and our own researchers (e.g. Hannan *et al.* 1991), individual manic or

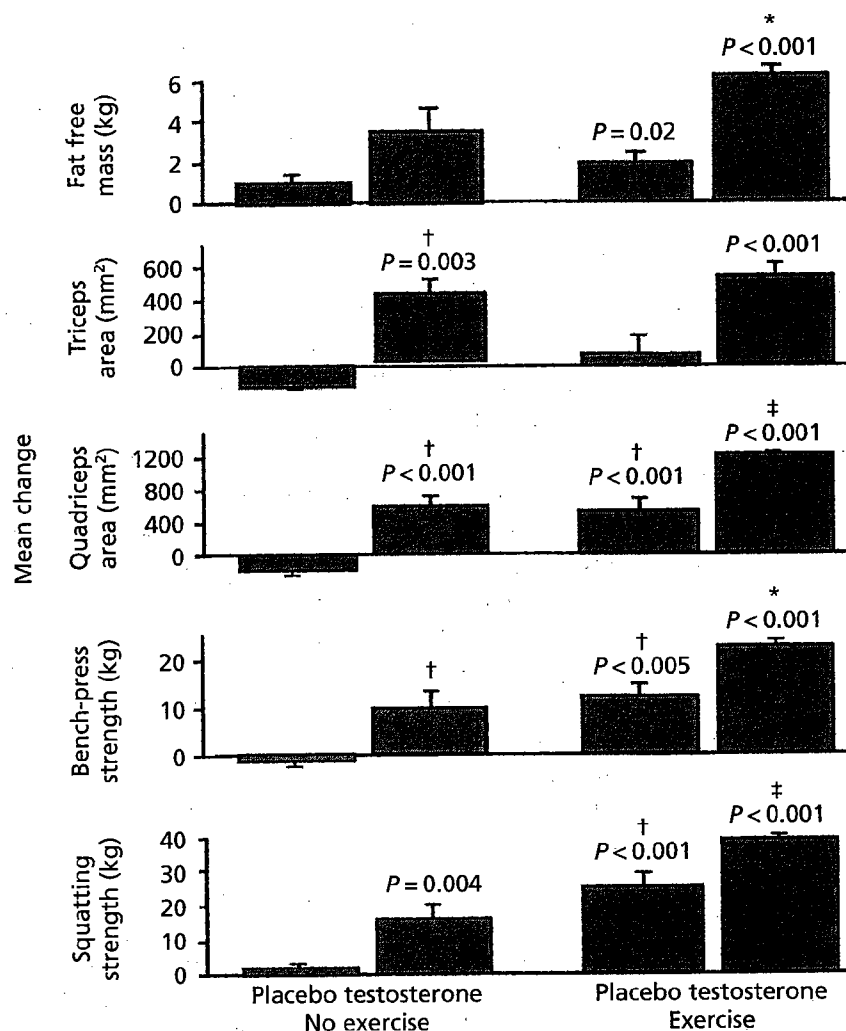


Fig. 35.4 Changes in body composition and strength measures in healthy young men administered placebo or 600 mg·wk⁻¹ testosterone enanthate for 20 weeks with or without an exercise program. (From Bhasin *et al.* 1996.)

hypomanic reactions have been observed, supporting the anecdotal case reports on manic behavior in androgen users. Pope *et al.* (2000) found significant responses in a specially designed test, the Point Subtraction Aggression Paradigm (PSAP), as well as the Young Mania Rating Scale and daily diary scores. The PSAP challenge test was particularly novel and appropriate in this study, probing individuals for a low threshold of aggressive response. A better understanding of who is at risk for the idiosyncratic reactions is vitally important to future human studies. Conceivably, the action is based on crossover activity to corticosteroid receptors (Janne 1990); psychotic episodes with high-dose corticosteroids have been well documented (Lewis & Smith 1983).

It is clear that almost irrational attitudes of invincibility provide a performance advantage, allowing athletes to withstand psychological fatigue and discomfort to persist and win. However, neurophysiologists have not completed the androgen connection between the behaviors, the neural circuits, and the biochemistry. Hannan *et al.* (1991) found an increase in dopamine metabolites in peripheral circulation of men administered high-dose androgens, suggesting the possibility of an interaction with the dopaminergic system, which has been implicated in a range of behaviors from highly focused, goal-directed behavior to psychosis. Indeed, androgens have been since demonstrated to increase HVA/DA ratios in the striatum of rats given one of four different androgens; methan-

dienone specifically increased dopamine synthesis (Thiblin *et al.* 1999). Hannan *et al.* (1990) had also shown in animal studies that stereotypic cage-exercising behavior was increased by androgen administration. Exercise effects on brain dopamine activity have been suggested to play a role in delaying exhaustion (Chaouloff *et al.* 1987) and might be interactive with androgens.

What do androgens do for endurance performance?

Androgens may provide endurance advantages through other non-psychological effects, including both energy availability and the oxygen-carrying capacity of the blood. These could be beneficial to performance of endurance athletes such as distance runners and cyclists. Earlier studies examined the effects of androgen supplementation on running and swimming performance. In an article that received high visibility in *Science*, Johnson and O'Shea (1969) reported that methandienone ($10 \text{ mg} \cdot \text{day}^{-1}$ for 3 weeks) increased maximal oxygen uptake by 15%, and significantly more than the gain in non-blinded control training partners. The same drug regimen failed to improve swim performance over that of a control group (O'Shea 1970), as did administration of oxandrolone ($10 \text{ mg} \cdot \text{day}^{-1}$ for 6 weeks) (O'Shea & Winkler 1970). Johnson *et al.* (1972) attempted to replicate the initial study reported in *Science*, measuring aerobic capacity after the same drug regimen of methandienone ($10 \text{ mg} \cdot \text{day}^{-1}$ for 3 weeks) or stanazolol ($6 \text{ mg} \cdot \text{day}^{-1}$ for 3 weeks), and a running training program, but found no change in maximal oxygen uptake or mile run time. Johnson speculated that the discrepancy in results could be explained by androgen-induced leg strength improvements that may have produced specific improvements in the bike test used in the original study, rather than a real increase in aerobic capacity. No further tests of androgens on aerobic performance in normal men have been reported.

There are plausible mechanisms to suggest androgen benefits to endurance performance. Androgens boost red blood cell production through stimulation of erythropoietin synthesis and secretion, direct stimulation of bone marrow hemato-

poiesis, and stimulation of iron incorporation into red blood cells. While androgen treatment has provided significant increases in hematocrit and hemoglobin in anemic patients (Neff *et al.* 1981), studies of high-dose androgen administration to normal men have not typically reported further increases (Mauss *et al.* 1975; Alen 1985; Kiraly 1988). However, Palacios *et al.* (1983) found a significant increase in red cell counts in normal men administered TE ($200 \text{ mg} \cdot \text{wk}^{-1}$ for 16 weeks), with modest increases in hematocrit and hemoglobin. Although rare, polycythemia has been reported in hypogonadal patients treated with androgens, usually in older patients. There may also be increases in blood volume (Wilson 1988), although this has not been verified in athletes. Thus, it is unclear that androgens boost hematological parameters in normal men, but if they did, there could be clear benefits to endurance performance. Erythrocyte infusions into normal men can substantially increase maximal oxygen uptake (Muza *et al.* 1987; Sawka *et al.* 1987). The downside is an increased risk of thromboembolytic injury such as stroke, especially with a potentiating effect of dehydration during intensive performance (Sawka *et al.* 1996). This is comparable to the risks that may be encountered by cyclists and other athletes using exogenous erythropoietin for performance enhancement (Adamson & Vapnek 1991).

Effects on the heart muscle and increases in stroke volume would theoretically also benefit endurance athletes. While the effects of androgens on left ventricular hypertrophy have been observed in strength athletes (e.g. McKillop *et al.* 1986), this has not been studied in endurance athletes, where steroid use appears to be less prevalent or far more covert.

It remains to be determined if androgens enhance lipolysis and energy availability for endurance performance. In one of the few studies to examine metabolic advantages, Guezennec *et al.* (1984a) tested endurance and metabolism of rats with elevated androgen levels during treadmill running for 7 h, but found no significant effects of testosterone other than a preservation of some of the glycogen stores. Other metabolic effects are potentially mediated through hormones such as IGF-I (Hobbs *et al.* 1993). Testosterone increases circulating IGF-I

levels, while nandrolone, which does not produce the same metabolites including estrogens, had no effect on total IGF-I levels. This suggests that there are differences in some of the biological actions of various synthetic anabolic steroids, determined by the receptor-binding affinities of the compounds and their metabolites. Androgens act to modify glucose disposal in normal men ($300 \text{ mg} \cdot \text{wk}^{-1}$ ND or TE) through non-insulin mechanisms (Hobbs *et al.* 1996).

Other hormonal responses to exercise, such as the acute rise in circulating growth hormone that occurs so dramatically in response to a serious strength athlete routine (more repetitions and short rests) compared to a body builder routine (higher weight and longer rests) (Kraemer *et al.* 1991), may also account for some of the effects currently ascribed to anabolic steroids. Growth hormone responds to acute metabolic signals, and like testosterone, specifically stimulates muscle protein and bone mineral accretion, and stimulates the release of IGF-I (Hindmarsh *et al.* 1997; Lee *et al.* 1997). It will be important to understand these interactions in the hormonal responses to exercise in future training studies in order to develop a systematic approach to science-based nutrition and training guidance.

Eunuchs may live longer, but do androgen-using athletes die sooner?

The health risks associated with androgen supplementation to normal men have been extensively reviewed and discussed elsewhere (e.g. Friedl 1990, 2000). In summary, the primary risks are a reversible reduction in sperm count and an accompanying testicular atrophy; hepatic adenoma that may be benign but may also result in hemorrhage and death; an adverse serum lipid profile with marked suppression of high-density lipoprotein (HDL)-cholesterol and unknown heart disease risk outcomes; changes in impulsivity and aggressiveness; skin changes including hair loss, oily skin, and acne; gynecomastia; and weight gain. Testosterone also plays a permissive role in prostate cancer, but a causative role remains to be demonstrated. From case reports of athletes using androgenic steroids, the majority of serious adverse health consequences

have been reported for bodybuilders. For example, in one review, 20 of the 28 case reports and most of the deaths involved bodybuilders (Friedl 2000).

Health benefits appear to accrue in replacement therapy in aging men, similar to hormone replacement therapy in women. These benefits include maintaining muscle mass and strength, which may be important in preventing injuries; maintenance of bone mass and prevention of male osteoporosis; antidepressant effects; and important effects on libido and motivation. Studies in the past decade indicate that androgens can increase glucose disposal separately from insulin-mediated mechanisms (Hobbs *et al.* 1996), as well as reduce intra-abdominal fat (Marin *et al.* 1995), both of which may be important in preventing type 2 diabetes. Reduction in fibrinolytic activity (Fearnley & Chakrabarti 1962) may reduce certain types of cardiovascular risks, perhaps countering effects such as the reduction in HDL-cholesterol, but conceivably increase hemorrhagic stroke risk. Although there is no defined disease of androgen excess in men, perhaps the effects of androgens are so ubiquitous that a wide variety of adverse health outcomes ranging from prostate cancer to heart disease are all diseases of androgen excess, just as being male is associated with shorter average lifespan. As a corollary of this, athletes that use androgenic steroids may be further trading lifespan for poorly defined performance advantages. Although eunuchs may have a longer average lifespan than normally virilized men (Hamilton 1948), it is unknown if the reverse is true. Epidemiological studies of retired athletes have been proposed but are fraught with complications, particularly in accurately ascertaining androgen administration histories and controlling for differences such as risk-taking behaviors in steroid users compared to non-steroid users.

Concern about the current increase in testosterone supplementation of older men at least has a tradeoff in disease prevention by reducing osteoporotic fractures and sarcopenia (that increases musculoskeletal injury risks). Replacement doses have been administered to many older men (> 45 years), and prescriptions for testosterone-replacement therapy have skyrocketed in the US in the past 5 years, providing a new cohort of men for studies

that are likely to provide new insights into the health consequences of androgen supplementation. There is also a growing database of experience with androgen administration to normal men from male contraceptive studies around the world. Other data on high-dose supplementation is emerging from efforts to sustain lean mass in muscular dystrophy and acquired immunodeficiency syndrome (AIDS) patients.

Conclusions

Androgenic steroids are banned at various levels of athletic competition, initially because of concerns about an uneven playing field, but then also out of concern for the health risks to the athletes. The greatest advantage is in bodybuilding in which huge muscle development is the goal, and the majority of case reports of serious health outcomes have centered on these athletes who are more likely to use very high doses that exceed levels that can be ethically studied. Other athletes appear to derive benefits from androgens for strength, endurance and overall psychological advantage. Prediction of the specific and individual effects of androgens is still uncertain. All of these compounds appear to have some positive musculotrophic effect, but the nature of this effect is still not well understood and almost certainly involves complex interaction with other hormones at the tissue level. Management of

diet and psychological stress are well-known factors in peak athletic performance, and it appears that endogenous testosterone levels can be markedly affected by these factors, making it a useful indicator of training status for males. The current thinking is that androgens may be useful in maintaining health and well-being in aging men as replacement therapy to prevent sarcopenia and osteoporosis. Supraphysiological doses that double or triple circulating levels have been tested in healthy young men for male contraception without remarkable effects other than modest weight gain. Higher doses, particularly when combined with exercise, may produce substantial gains in muscle mass and strength. Anecdotal reports of the use of extraordinarily high levels of androgenic steroids that extend well beyond the observations in controlled studies, involve primarily body builders and are associated with the majority of case reports of serious adverse health effects.

Acknowledgements

The author gratefully acknowledges the editorial assistance of Ms. Shari Hallas and technical assistance provided by Sergeant Joseph Alemany.

The opinions and assertions in this chapter are those of the author and do not necessarily represent the official views or policies of the Army or the Department of Defense.

References

- Aakvaag, A., Bentdal, O., Quigstad, K. *et al.* (1978) Testosterone and testosterone binding globulin (TeBG) in young men during prolonged stress. *International Journal of Andrology* **1**, 22–31.
- Adamson, J.W. & Vapnek, D. (1991) Recombinant erythropoietin to improve athletic performance. *New England Journal of Medicine* **324**, 698–699.
- Aguilera, R., Hatton, C.K. & Catlin, D.H. (2002) Detection of epitestosterone doping by isotopic ratio mass spectrometry. *Clinical Chemistry* **48**, 629–636.
- Alen, M. (1985) Androgenic steroid effects on liver and red cells. *British Journal of Sports Medicine* **19**, 15–20.
- Alen, M., Häkkinen, K. & Komi, P.V. (1984) Changes in neuromuscular performance and muscle fiber characteristics of elite power athletes self-administering androgenic and anabolic steroids. *Acta Physiologica Scandinavica* **122**, 535–544.
- Antonio, J., Wilson, J.D. & George, F.W. (1999) Effects of castration and androgen treatment on androgen-receptor levels in rat skeletal muscles. *Journal of Applied Physiology* **87**, 2016–2019.
- Ariel, G. & Saville, W. (1972) Effect of anabolic steroids on reflex components. *Journal of Applied Physiology* **32**, 795–797.
- Bagatell, C.J. & Bremner, W.J. (1990) Sperm counts and reproductive hormones in male marathoners and lean controls. *Fertility and Sterility* **53**, 688–692.
- Bambino, T.H. & Hsueh, A.J.W. (1981) Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis *in vivo* and *in vitro*. *Endocrinology* **108**, 2142–2148.
- Bardin, C.W. & Catterall, J.F. (1981) Testosterone: a major determinant of extragenital sexual dimorphism. *Science* **211**, 1285–1294.
- Bardin, C.W., Catterall, J.F. & Janne, O.A. (1990) The androgen-induced phenotype. *Anabolic Steroid Abuse, Research Monograph* **102**, 131–141.
- Behre, H.M. & Nieschlag, E. (1992) Testosterone buciclate (20 Aet-1) in hypogonadal men: pharmacokinetics and pharmacodynamics of the new long-acting androgen ester. *Journal of Clinical Endocrinology and Metabolism* **75**, 1204–1210.

- Bhasin, S., Storer, T.W., Berman, N. *et al.* (1996) The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *New England Journal of Medicine* 335, 1–7.
- Bhasin, S., Storer, T.W., Berman, N. *et al.* (1997) Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. *Journal of Clinical Endocrinology and Metabolism* 82, 407–413.
- Bhasin, S., Woodhouse, L., Casaburi, R. *et al.* (2001) Testosterone dose–response relationships in healthy young men. *American Journal of Physiology* 281, E1172–E1181.
- Blanchard, D.C., Sakai, R.R., McEwen, B., Weiss, S.M. & Blanchard, R.J. (1993) Subordination stress: behavioral, brain, and neuroendocrine correlates. *Behavior and Brain Research* 58, 113–121.
- Bourne, P.G., Rose, R.M. & Mason, J.W. (1968) 17-OHCS levels in combat: Special Forces 'A' team under threat of attack. *Archives of General Psychiatry* 19, 5–14.
- Catlin, D.H., Ahrens, B.D. & Kucherova, Y. (2002) Detection of norbolethone, an anabolic steroid never marketed, in athletes' urine. *Rapid Communications in Mass Spectrometry* 16, 1273–1275.
- Chaouloff, F., Laude, D., Merino, D. *et al.* (1987) Amphetamine and α -methyl-p-tyrosine affect the exercise-induced imbalance between the availability of tryptophan and synthesis of serotonin in the brain of the rat. *Neuropharmacology* 26, 1099–1106.
- Collu, R., Gibb, W. & Ducharme, J.R. (1984) Role of catecholamines in the inhibitory effect of immobilization stress on testosterone secretion in rats. *Biology of Reproduction* 30, 416–422.
- Crawford, B.A., Liu, P.Y., Kean, M.T., Bleasel, J.F. & Handelsman, D.J. (2003) Randomized placebo-controlled trial of androgen effects on muscle and bone in men requiring long-term systemic glucocorticoid treatment. *Journal of Clinical Endocrinology and Metabolism* 88, 3167–3176.
- Crist, D.M., Stackpole, P.J. & Peake, G.T. (1983) Effects of androgenic-anabolic steroids on neuromuscular power and body composition. *Journal of Applied Physiology* 54, 366–370.
- Cumming, D.C., Brunsting, L.A., Strich, G., Ries, A.L. & Rebar, R.W. (1986) Reproductive hormone increases in response to acute exercise in men. *Medicine and Science in Sports and Exercise* 18, 369–373.
- De Souza, M.J., Arce, J.C., Pescatello, L.S., Scherzer, H.S. & Luciano, A.A. (1994) Gonadal hormones and semen quality in male runners. A volume threshold effect of endurance training. *International Journal of Sports Medicine* 15, 383–391.
- Dessypris, A., Kuoppasalmi, K. & Adlercreutz, H. (1976) Plasma cortisol, testosterone, androstenedione, and luteinizing hormone in a non-competitive marathon run. *Journal of Steroid Biochemistry* 7, 31–37.
- Elbers, J.M., Asscheman, H., Seidell, J.C., Megens, J.A. & Gooren, L.J. (1997) Long-term testosterone administration increases visceral fat in female-to-male transsexuals. *Journal of Clinical Endocrinology and Metabolism* 82, 2044–2047.
- Elbers, J.M., Asscheman, H., Seidell, J.C. & Gooren, L.J. (1999) Effects of sex steroid hormones on regional fat depots as assessed by magnetic resonance imaging in transsexuals. *American Journal of Physiology* 276, E317–E325.
- Elias, M. (1981) Serum cortisol, testosterone, and testosterone-binding globulin responses to competitive fighting in human males. *Aggressive Behavior* 7, 215–224.
- Era, P., Alen, M. & Rahkila, P. (1988) Psychomotor and motor speed in power athletes self-administering testosterone and anabolic steroids. *Research Quarterly for Exercise and Sport* 59, 50–56.
- Fearnley, G.R. & Chakrabarti, R. (1962) Increase of blood fibrinolytic activity by testosterone. *Lancet* ii, 128–132.
- Forbes, G.B. (1985) The effect of anabolic steroids on lean body mass: the dose–response curve. *Metabolism* 34, 571–573.
- Forbes, G.B. (1993) The companionship of lean and fat. In: *Human Body Composition* (Ellis, K.J. & Eastman, J.D., eds.). Plenum Press, New York: 1–14.
- Forbes, G.B., Porta, C.R., Herr, B.E. & Griggs, R.C. (1992) Sequence of changes in body composition induced by testosterone and reversal of changes after drug is stopped. *Journal of the American Medical Association* 267, 397–399.
- Francis, K.T. (1981) The relationship between high and low trait psychological stress, serum testosterone, and serum cortisol. *Experientia* 37, 1296–1297.
- Freed, D.J.L., Banks, A.J., Longson, D. & Burley, D.M. (1975) Anabolic steroids in athletics: crossover double-blind trial on weightlifters. *British Medical Journal* 2, 471–473.
- Friedl, K.E. (1990) Reappraisal of the health risks associated with the use of high doses of oral and injectable androgenic steroids. *National Institute on Drug Abuse Research Monograph* 102, 142–177.
- Friedl, K.E. (1997) Variability of fat and lean tissue loss during physical exertion with energy deficit. In: *Physiology, Stress and Malnutrition: Functional Correlates, Nutritional Intervention* (Kinney, J.M. & Tucker, H.N., eds.). Lippincott-Raven Publishers, New York: 431–450.
- Friedl, K.E. (2000) Effect of anabolic steroid use on body composition and physical performance. In: *Anabolic Steroids in Sport and Exercise*, 2nd edn (Yesalis, C.E., ed.). Human Kinetics Publishers, Champaign, IL: 139–174.
- Friedl, K.E. (2004) Can you be large and not obese? The distinction between body weight, body fat, and abdominal fat in occupational standards. *Diabetes Technology and Therapeutics* 6, 732–749.
- Friedl, K.E. & Yesalis, C.E. (1989) Self-treatment of gynecomastia in bodybuilders who use anabolic steroids. *Physician and Sportsmedicine* 17, 67–79.
- Friedl, K.E., Hannan, C.J., Jr., Jones, R.E. & Plymate, S.R. (1990) HDL-cholesterol is not decreased if an aromatizable androgen is administered. *Metabolism* 39, 69–74.
- Friedl, K.E., Dettori, J.R., Hannan, C.J., Jr., Patience, T.H. & Plymate, S.R. (1991) Comparison of the effects of high dose testosterone and 19-nortestosterone to a replacement dose of testosterone on strength and body composition in normal men. *Journal of Steroid Biochemistry and Molecular Biology* 40, 607–612.
- Friedl, K.E., DeLuca, J.P., Marchitelli, L.J. & Vogel, J.A. (1992a) Reliability of body-fat estimations from a four-compartment model using density, body water, and bone mineral measurements. *American Journal of Clinical Nutrition* 55, 764–770.
- Friedl, K.E., Moore, R.J. & Marchitelli, L.J. (1992b) Steroid replacers: let the athlete beware. *National Strength and Conditioning Association Journal* 14, 14–19.
- Friedl, K.E., Moore, R.J., Martinez-Lopez, L.E. *et al.* (1994) Lower limits of body fat in healthy active men. *Journal of Applied Physiology* 77, 933–940.
- Friedl, K.E., Moore, R.J., Hoyt, R.W. *et al.* (2000) Endocrine markers of semistarvation in healthy lean men in a multistressor environment. *Journal of Applied Physiology* 88, 1820–30.

- Friedl, K.E., Mays, M.Z., Kramer, T.R. & Shippee, R.L. (2001) Acute recovery of physiological and cognitive function in US Army Ranger students in a multistressor field environment. In: *The Effect of Prolonged Military Activities in Man—Physiological and Biochemical Changes—Possible Means of Rapid Recuperation*. Technical Report RTO-MP-042. Research and Technological Organization, North Atlantic Treaty Organization, Neuilly-sur-Seine Cedex, France: 6.1–6.10.
- Gallagher, D., Heymsfield, S.B. & Wang, Z.M. (1999) Skeletal muscle markers. In: *Protein and Amino Acids*. National Academy Press, Washington, DC: 255–277.
- Glass, A.R. & Vigersky, R.A. (1980) Resensitization of testosterone production in men after human chorionic gonadotropin-induced desensitization. *Journal of Clinical Endocrinology and Metabolism* **51**, 1395–1400.
- Griggs, R.C., Kingston, W., Jozefowicz, R.F. *et al.* (1989) Effect of testosterone on muscle mass and muscle protein synthesis. *Journal of Applied Physiology* **66**, 498–503.
- Guezennec, C.Y., Ferre, P., Serrurier, B. *et al.* (1984a) Metabolic effects of testosterone during prolonged physical exercise and fasting. *European Journal of Applied Physiology* **52**, 300–304.
- Guezennec, C.Y., Serrurier, B., Aymonod, M., Merino, D. & Pesquies, P.C. (1984b) Metabolic and hormonal response to short term fasting after endurance training in the rat. *Hormone and Metabolic Research* **16**, 572–575.
- Guezennec, Y., Leger, L., Lhoste, F., Aymonod, M. & Pesquies, P.C. (1986) Hormone and metabolite response to weight-lifting training sessions. *International Journal of Sports Medicine* **7**, 100–105.
- Guezennec, C.Y., Satabin, P., Legrand, H. & Bigard, A.X. (1994) Physical performance and metabolic changes induced by combined prolonged exercise and different energy intakes in humans. *European Journal of Applied Physiology* **68**, 525–530.
- Gustafsson, J.A., Saartok, T., Dahlberg, E. *et al.* (1984) Studies on steroid receptors in human and rabbit skeletal muscle: clues to the understanding of the mechanism of action of anabolic steroids. *Progress in Clinical and Biological Research* **142**, 261–290.
- Hamilton, J.B. (1948) The role of testicular secretions as indicated by the effects of castration in man and by studies of pathological conditions and the short lifespan associated with maleness. *Recent Progress in Hormone Research* **3**, 257–322.
- Hannan, C.J., Jr., Friedl, K.E. & Plymate, S.R. (1990) Effect of testosterone on apomorphine induced movements in the rat. *Workshop Conference on Androgen Therapy: Biologic and Clinical Consequences*, 17–20 January 1990, Marco Island, FL.
- Hannan, C.J., Jr., Friedl, K.E., Kettler, T.M. & Plymate, S.R. (1991) Psychological and serum homovanillic acid changes in men administered androgenic steroids. *Psychoneuroendocrinology* **16**, 335–43.
- Hartgens, F., Van Marken Lichtenbelt, W.D., Ebbing, S. *et al.* (2001) Body composition and anthropometry in body builders: regional changes due to nandrolone decanoate administration. *International Journal of Sports Medicine* **22**, 235–241.
- Haupt, H.A. & Rovere, G.D. (1984) Anabolic steroids: a review of the literature. *American Journal of Sports Medicine* **12**, 469–484.
- Hellhammer, D.H., Hubert, W. & Schurmeyer, T. (1985) Changes in saliva testosterone after psychological stimulation in men. *Psychoneuroendocrinology* **10**, 77–81.
- Hershberger, L.G., Shipley, E.G. & Meyer, R.K. (1953) Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. *Proceedings of the Society for Experimental Biology and Medicine* **83**, 175–180.
- Hervey, G.R., Hutchinson, I., Knibbs, A.V. *et al.* (1976) 'Anabolic' effects of methandienone in men undergoing athletic training. *Lancet* **2**, 699–702.
- Hervey, G.R., Knibbs, A.V., Burkinshaw, L. *et al.* (1981) Effects of methandienone on the performance and body composition of men undergoing athletic training. *Clinical Science* **60**, 457–461.
- Hickson, R.C., Kurowski, T.T., Capaccio, J.A. & Chatterton, R.T., Jr. (1984) Androgen cytosol binding in exercise-induced sparing of muscle atrophy. *American Journal of Physiology* **247**, E597–E603.
- Hindmarsh, P.C., Fall, C.H.D., Pringle, P.J., Osmond, C. & Brook, C.G.D. (1997) Peak and trough growth hormone concentrations have different associations with the insulin-like growth factor axis, body composition, and metabolic parameters. *Journal of Clinical Endocrinology and Metabolism* **82**, 2172–2176.
- Hobbs, C.J., Plymate, S.R., Rosen, C.J. & Adler, R.A. (1993) Testosterone administration increases insulin-like growth factor I levels in normal men. *Journal of Clinical Endocrinology and Metabolism* **77**, 776–779.
- Hobbs, C.J., Jones, R.E. & Plymate, S.R. (1996) Nandrolone, a 19-nortestosterone, enhances insulin-independent glucose uptake in normal men. *Journal of Clinical Endocrinology and Metabolism* **81**, 1582–1585.
- Houmard, J.A., McCulley, C., Shinebarger, M.H. & Bruno, N.J. (1994) Effects of exercise training on plasma androgens in men. *Hormone and Metabolism Research* **26**, 297–300.
- Hughes, B.J. & Krieg, M. (1988) Steroid receptors and the muscular system. In: *Steroid Receptors and Disease: Cancer, Autoimmune, Bone, and Circulatory Disorders* (Sheridan, P.J., Blum, K. & Trachtenberg, M.C., eds.). Marcel Dekker, New York: 415–433.
- Janne, O.A. (1990) Androgen interaction through multiple steroid receptors. *National Institute on Drug Abuse Research Monograph* **102**, 178–186.
- Jezova, D. & Vigas, M. (1981) Testosterone response to exercise during blockade and stimulation of adrenergic receptors in man. *Hormone Research* **15**, 141–147.
- Jezova-Repecikova, D., Vigas, M., Miklaj, L. & Jurcovicova, J. (1982) Plasma testosterone during bicycle ergometer exercise without and after L-dopa pretreatment. *Endocrinologia Experimentalis*, **16**, 3–8.
- Johnson, L.C. & O'Shea, J.P. (1969) Anabolic steroid: effects on strength development. *Science* **164**, 957–959.
- Johnson, L.C., Fisher, G., Silvester, L.J. & Hofheins, C.C. (1972) Anabolic steroid: effects on strength, body weight, oxygen uptake and spermatogenesis upon mature males. *Medicine and Science in Sports* **4**, 43–45.
- King, D.S., Sharp, R.L., Vukovich, M.D. *et al.* (1999) Effect of oral androstenedione on serum testosterone and adaptations to resistance training in young men: a randomized controlled trial. *Journal of the American Medical Association* **281**, 2020–2028.
- Kiraly, C.L. (1988) Androgenic-anabolic steroid effects on serum and skin surface lipids, on red cells, and on liver

- enzymes. *International Journal of Sports Medicine* 9, 249–252.
- Knight, J. (2003) Drugs bust reveals athletes' secret steroid. *Nature* 425, 752.
- Kochakian, C.D. (1988) The evolution from 'the male hormone' to anabolic-androgenic steroids. *Alabama Journal of Medical Sciences* 25, 96–102.
- Kouri, E.M., Lukas, S.E., Pope, H.G. & Oliva, P. (1995a) Increased aggressive responding in male volunteers following the administration of gradually increasing doses of testosterone cypionate. *Drug and Alcohol Dependence* 40, 73–79.
- Kouri, E.M., Pope, H.G., Katz, D.L. & Oliva, P. (1995b) Fat-free mass index in users and nonusers of anabolic-androgenic steroids. *Clinical Journal of Sports Medicine* 5, 223–228.
- Kraemer, W.J., Gordon, S.E., Fleck, S.J. *et al.* (1991) Endogenous anabolic hormone and growth factor responses to heavy resistance exercise in males and females. *International Journal of Sports Medicine* 12, 228–35.
- Kreuz, L.E., Rose, R.M. & Jennings, J.R. (1972) Suppression of plasma testosterone levels and psychological stress. A longitudinal study of young men in Officer Candidate School. *Archives of General Psychiatry* 26, 479–482.
- Kruskemper, H.L. (1968) *Anabolic Steroids*. Academic Press, New York.
- Kuipers, H., Binkhorst, F.P., Hartgens, F., Wijnen, J.A. & Keizer, H.A. (1993) Muscle ultrastructure after strength training with placebo or anabolic steroid. *Canadian Journal of Applied Physiology* 18, 189–196.
- Kuoppasalmi, K., Naveri, H., Harkonen, M. & Adlerkreuz, H. (1980) Plasma cortisol, androstenedione, testosterone, and luteinizing hormone in running exercise of different intensities. *Scandinavian Journal of Clinical and Laboratory Investigation* 40, 403–409.
- Lee, D.K.P. (2002) Androgen receptor enhances myogenin expression and accelerates differentiation. *Biochemical and Biophysical Research Communications* 294, 408–413.
- Lee, D.K.P., Durham, S.K., Martinez, V. *et al.* (1997) Kinetics of insulin-like growth factor (IGF) and IGF-binding protein responses to a single dose of growth hormone. *Journal of Clinical Endocrinology and Metabolism* 82, 2266–2274.
- Lewis, D.A. & Smith, R.E. (1983) Steroid-induced psychiatric syndromes—a report of 14 cases and a review of the literature. *Journal of Affective Disorders* 5, 319–332.
- Liddle, G.W. & Burke, H.A. (1960) Anabolic steroids in clinical medicine. *Helvetica Medica Acta* 27, 504–513.
- Loucks, A.B. (2004) Energy balance and body composition in sports and exercise. *Journal of Sports Sciences* 22, 1–14.
- Loucks, A.B. & Thuma, J.R. (2003) Luteinizing hormone pulsatility is disrupted at a threshold of energy availability in regularly menstruating women. *Journal of Clinical Endocrinology and Metabolism* 88, 297–311.
- Lucia, A., Chicharro, J.L., Perez, M. *et al.* (1996) Reproductive function in male endurance athletes: sperm analysis and hormonal profile. *Journal of Applied Physiology* 81, 2627–2636.
- McKillop, G., Todd, I.C. & Ballantyne, D. (1986) Increased left ventricular mass in a bodybuilder using steroids. *British Journal of Sports Medicine* 20, 151–152.
- Marin, P. (1995) Testosterone and regional fat distribution. *Obesity Research* 3, 609S–612S.
- Marin, P., Lonn, L., Andersson, B. *et al.* (1996) Assimilation of triglycerides in subcutaneous and intraabdominal adipose tissue *in vivo* in men: effects of testosterone. *Journal of Clinical Endocrinology and Metabolism* 81, 1018–1022.
- Mauras, N., Hayes, V., Welch, S. *et al.* (1998) Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. *Journal of Clinical Endocrinology and Metabolism* 83, 1886–1892.
- Mauss, J., Borsch, G., Bormacher, K. *et al.* (1975) Effect of a long-term testosterone oenanthate administration on male reproductive function: clinical evaluation, serum FSH, LH, testosterone, and seminal fluid analyses in normal men. *Acta Endocrinologica* 78, 373–384.
- Michel, G. & Baulieu, E.E. (1980) Androgen receptor in rat skeletal muscle: characterization and physiological variations. *Endocrinology* 107, 2088–2097.
- Morville, R., Pesquies, P.C., Guezennec, C.Y., Serrurier, B.D. & Guignard, M. (1979) Plasma variations in testicular and adrenal androgens during prolonged physical exercise in man. *Annales d'Endocrinologie* 40, 501–510.
- Muza, S.R., Sawka, M.N., Young, A.J. *et al.* (1987) Elite special forces: physiological description and ergogenic influence of blood reinfusion. *Aviation Space and Environmental Medicine* 58, 1001–1004.
- Neff, M.S., Goldberg, J., Slifkin, R.F. *et al.* (1981) A comparison of androgens for anemia in patients on hemodialysis. *New England Journal of Medicine* 304, 871–875.
- O'Connell, M., Robbins, D.C., Horton, E.S., Sims, E.A.H. & Danforth, E., Jr. (1979) Changes in serum concentrations of 3,3',5'-triiodothyronine and 3,5,3'-triiodothyronine during prolonged moderate exercise. *Journal of Clinical Endocrinology and Metabolism* 49, 242–246.
- Opstad, P.K. (1992a) Androgenic hormones during prolonged physical stress, sleep, and energy deficiency. *Journal of Clinical Endocrinology and Metabolism* 74, 1176–1183.
- Opstad, P.K. (1992b) The hypothalamo-pituitary regulation of androgen secretion in young men after prolonged physical stress combined with energy and sleep deprivation. *Acta Endocrinologica* 127, 231–236.
- O'Shea, J.P. (1970) Anabolic steroid: effects on competitive swimmers. *Nutrition Report International* 1, 337–342.
- O'Shea, J.P. & Winkler, W. (1970) Biochemical and physical effects of an anabolic steroid in competitive swimmers and weightlifters. *Nutrition Reports International* 2, 351–362.
- Palacios, A., Campfield, L.A., McClure, R.D., Steiner, B. & Swerdloff, R.S. (1983) Effect of testosterone enanthate on hematopoiesis in normal men. *Fertility and Sterility* 40, 100–104.
- Paul, A.C. & Rosenthal, N. (2002) Different modes of hypertrophy in skeletal muscle fibers. *Journal of Cell Biology* 156, 751–760.
- Ponjee, G.A., DeRooy, H.A. & Vader, H.L. (1994) Androgen turnover during marathon running. *Medicine and Science in Sports and Exercise* 26, 1274–1277.
- Pope, H.G., Jr. & Katz, D.L. (1988) Affective and psychotic symptoms associated with anabolic steroid use. *American Journal of Psychiatry* 145, 487–490.
- Pope, H.G., Jr. & Katz, D.L. (1994) Psychiatric and medical effects of anabolic-androgenic steroid use. *Archives of General Psychiatry* 51, 375–382.
- Pope, H.G., Jr., Kouri, E.M. & Hudson, J.I. (2000) Effects of supraphysiologic doses of testosterone on mood and aggression in normal men. *Archives of General Psychiatry* 57, 133–140.
- Roemmich, J.M. & Sinning, W.E. (1997) Weight loss and wrestling training: effects on growth-related hormones.

- Journal of Applied Physiology* 82, 1760–1764.
- Rogol, A.D., Veldhuis, J.D., Williams, F.A. & Johnson, M.L. (1984) Pulsatile secretion of gonadotropins and prolactin in male marathon runners—relation to the endogenous opiate system. *Journal of Andrology* 5, 21–27.
- Rowell, L.B., Blackmon, J.R. & Bruce, R.A. (1964) Indocyanine green clearance and estimated hepatic blood flow during mild to maximal exercise in upright man. *Journal of Clinical Investigation* 43, 1677–1690.
- Samuels, L.T., Henschel, A.F. & Keys, A. (1942) Influence of methyl testosterone on muscular work and creatine metabolism in normal young men. *Journal of Clinical Endocrinology* 2, 649–654.
- Sapolsky, R.M. (1985) Stress-induced suppression of testicular function in the wild baboon: role of glucocorticoids. *Endocrinology* 116, 2273–2278.
- Sapolsky, R.M. (1986) Stress-induced elevation of testosterone concentrations in high ranking baboons: role of catecholamines. *Endocrinology* 118, 1630–1635.
- Saris, W.H.M., van Erp-Baart, M.A., Brouns, F., Westerterp, K.R. & ten Hoor, F. (1989) Study of food intake and energy expenditure during extreme sustained exercise: the Tour de France. *International Journal of Sports Medicine* 10, S26–S31.
- Sawka, M.N., Young, A.J., Muza, S.R., Gonzalez, R.R. & Pandolf, K.B. (1987) Erythrocyte reinfusion and maximal aerobic power. An examination of modifying factors. *Journal of the American Medical Association* 257, 1496–1499.
- Sawka, M.N., Joyner, M.J., Miles, D.S. et al. (1996) American College of Sports Medicine position stand. The use of blood doping as an ergogenic aid. *Medicine and Science in Sports and Exercise* 28, i–viii.
- Schmid, P., Pusch, H.H., Wolf, W. et al. (1982) Serum FSH, LH, and testosterone in humans after physical exercise. *International Journal of Sports Medicine* 3, 84–89.
- Schroeder, E.T., Terk, M. & Sattler, F.R. (2003) Androgen therapy improves muscle mass and strength but not muscle quality: results from two studies. *American Journal of Physiology* 285, E16–E24.
- Schurmeyer, T., Wickings, E.J., Freischerm, C.W. & Nieschlag, E. (1983) Saliva and serum testosterone following oral testosterone undecanoate administration in normal and hypogonadal men. *Acta Endocrinologica* 102, 456–462.
- Schurmeyer, T., Jung, K. & Nieschlag, E. (1984) The effect of an 1100 km run on testicular, adrenal and thyroid hormones. *International Journal of Andrology* 7, 276–282.
- Sinha-Hikim, I., Artaza, J., Woodhouse, L. et al. (2002) Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. *American Journal of Physiology* 283, E154–E164.
- Stanhope, R., Buchanan, C.R., Fenn, G.C. & Preece, M.A. (1988) Double blind placebo controlled trial of low dose oxandrolone in the treatment of boys with constitutional delay of growth and puberty. *Archives of Disease in Childhood* 63, 501–505.
- Storer, T.W., Magliano, L., Woodhouse, L. et al. (2003) Testosterone dose-dependently increases maximal voluntary strength and leg power, but does not affect fatigability or specific tension. *Journal of Clinical Endocrinology and Metabolism* 88, 1478–1485.
- Strauss, R.H., Lanese, R.R. & Malarkey, W.B. (1985) Weight loss in amateur wrestlers and its effect on serum testosterone levels. *Journal of the American Medical Association* 254, 3337–3338.
- Sundaram, K., Kumar, N., Monder, C. & Bardin, C.W. (1995) Different patterns of metabolism determine the relative anabolic activity of 19-norandrogens. *Journal of Steroid Biochemistry and Molecular Biology* 53, 253–257.
- Sutton, J.R., Coleman, M.J., Casey, J. & Lazarus, L. (1973) Androgen responses during physical exercise. *British Medical Journal* 1, 520–522.
- Thiblin, L., Finn, A., Ross, S.B. & Stenfors, C. (1999) Increased dopaminergic and 5-hydroxytryptaminergic activities in male rat brain following long-term treatment with anabolic androgenic steroids. *British Journal of Pharmacology* 126, 1301–1306.
- Ursine, H., Baade, E. & Levine, S. (eds.) (1978) *Psychobiology of Stress. A Study of Coping Men*. Academic Press, New York.
- Viru, A.A. (1985) *Hormones in Muscular Activity. I. Hormonal Ensemble in Exercise*. CRC Press, Boca Raton, FL: 87.
- Welle, S., Jozefowicz, R., Forbes, G. & Grigg, R.C. (1992) Effect of testosterone on metabolic rate and body composition of normal men and men with muscular dystrophy. *Journal of Clinical Endocrinology and Metabolism* 74, 332–335.
- Wheeler, G.D., Wall, S.R., Belcastro, A.N. & Cumming, D.C. (1984) Reduced serum testosterone and prolactin levels in male distance runners. *Journal of the American Medical Association* 252, 514–516.
- Wilkerson, J., Horvath, S. & Gutin, B. (1980) Plasma testosterone during treadmill exercise. *Journal of Applied Physiology* 49, 249–253.
- Wilson, J.D. (1988) Androgen abuse by athletes. *Endocrine Reviews* 9, 181–198.
- Wren, K., Zhang, X.W., Toombs, A., Harada, S. & Jepsen, K. (2003) Targeted overexpression of androgen receptor in osteoblasts results in complex skeletal phenotype in growing animals. *Journal of Bone and Mineral Research* 18, S293 [Abstract].
- Woodhouse, L.J., Gupta, H., Bhasin, M. et al. (2004) Dose-dependent effects of testosterone on regional adipose tissue distribution in healthy young men. *Journal of Clinical Endocrinology and Metabolism* 89, 718–726.
- World Health Organization Task Force on Methods for the Regulation of Male Fertility (1990) Contraceptive efficacy of testosterone-induced azoospermia in normal men. *Lancet* 336, 955–959.
- Wright, J.E. (1980) Anabolic steroids and athletics. *Exercise and Sport Sciences Reviews* 8, 149–202.
- Yesalis, C.E., III, (ed.) (2000) *Anabolic Steroids in Sport and Exercise*, 2nd edn. Human Kinetics, Champaign, IL.
- Yesalis, C.E., III, Herrick, R.T., Buckley, W.E. et al. (1988) Self-reported use of anabolic-androgenic steroids by elite power lifters. *Physician and Sportsmedicine* 16, 90–100.
- Young, N.R., Baker, H.W., Liu, G. & Seeman, E. (1993) Body composition and muscle strength in healthy men receiving testosterone enanthate for contraception. *Journal of Clinical Endocrinology and Metabolism* 77, 1028–1032.

**ESPN.com:** Cycling[\[Print without images\]](#)

Thursday, July 27, 2006

Doping expert thinks Landis result 'doesn't add up'

Associated Press

Tour de France champion Floyd Landis' results on a urine test that spots elevated levels of performance-enhancing testosterone are a mystery and "don't add up," a leading doping expert said Thursday.

Landis' team revealed Thursday that his urine sample last week showed "an unusual level of testosterone/epitestosterone."

Testosterone creams, pills and injections can build muscle and strength and improve recovery time after exertion when used over a period of several weeks, according to Dr. Gary Wadler, a member of the World Anti-Doping Agency and a spokesman for the American College of Sports Medicine.

But if Landis had been a user, earlier urine tests during the Tour would have been affected, too, Wadler said. Landis' first reported abnormal result was last Thursday, after his amazing come-from-behind performance in stage 17 of the race.

One-time use of steroids could result in an abnormal test, but it would have no effect on performance and could not account for Landis' astounding feat Thursday, "so something's missing here," Wadler said. "It just doesn't add up."

The test detects both testosterone and a related steroid called epitestosterone, which is not performance-enhancing. Both are produced by the body and are also made in synthetic form.

The usual ratio for both substances is about 1:1 or 2:1, Wadler said.

Suspicions for improper steroid use arise when the ratio climbs above 4 parts testosterone over 1 part epitestosterone, Wadler said. Officials have not said what ratio Landis' test showed.

Athletes who use performance-enhancing anabolic steroids often also take synthetic epitestosterone to equalize the ratio, said Charles Yesalis, a recently retired Pennsylvania State University professor and doping expert.

There is no medical use for synthetic epitestosterone; it is used "to cheat drug tests," Yesalis said.

Some men have naturally occurring high levels of testosterone and/or epitestosterone, but there is a sophisticated lab test called a carbon isotope ratio test that is often used to detect synthetic forms.

Alcohol can influence testosterone-epitestosterone levels, but more often in women than in men and it would be unlikely to have a huge effect, Wadler said.

Landis said in an interview during the Tour de France that he has had injections of cortisone, a medically used steroid drug to treat pain from a degenerating arthritic hip, but doctors said that would not affect his test results.

Corticosteroids "have zero impact" on the testosterone-epitestosterone test, Wadler said.

Expert's Q&A

In an exclusive interview with ESPN.com, Dr. Gary Wadler says that it's "extraordinarily unusual" for a "B" sample to exonerate a rider but that Floyd Landis' test results still don't make sense.

• For more of from Wadler, [Click here.](#)

GDC0054

The typical procedure for urine-testing of athletes involves taking two samples at the same time and bottling them separately. The "A" sample is tested first, and if it is normal the "B" sample is discarded. If the "A" sample shows elevated testosterone levels, the "B" sample is tested, and its results are used to confirm use of a banned substance, Wadler said.

The same "B" sample is also often subjected to the carbon isotope test, said Dr. Don Catlin, director of a World Anti-Doping Association-accredited Olympic lab at UCLA.

Landis' Phonak team suspended him pending results of the backup "B" sample.

ESPN.com: [Help](#) | [PR Media Kit](#) | [Sales Media Kit](#) | [Report a Bug](#) | [Contact Us](#) | [Site Map](#) | [Jobs at ESPN](#) | [Supplier Information](#) | Copyright ©2007 ESPN Internet Ventures. [Terms of Use](#) and [Privacy Policy](#) and [Safety Information/Your California Privacy Rights](#) are applicable to you. All rights reserved.

Written Statement

GARY I. WADLER, M.D., FACP, FACSM, FACPM, FCP
Associate Professor of Clinical Medicine
New York University School of Medicine

Hearing on
Major League Baseball and the Use of performance-Enhancing Drugs

Before the
U.S. Congress House of Representatives Committee on Government Reform

March 17, 2005

Dear Mr. Chairman and Members of the Committee:

I am honored to appear here today and I appreciate the opportunity to testify. My name is Dr. Gary I. Wadler, and I am an Associate Professor of Clinical Medicine at New York University School of Medicine, a Fellow and former Trustee of the largest sports medicine and exercise science organization in the world, the American College of Sports Medicine, where I had chaired its Health and Science Policy Committee. In addition, I am a member of the Board of Stewards and former Vice-President of Women's Sports Foundation. I have served as a member of the Technical Advisory Committee of the CASA National Commission on Sports and Substance Abuse. At the local level, I serve as Chairman and President of the Nassau County Sports Commission.

I am the lead author of the internationally acclaimed text, *Drugs and the Athlete*, and the recipient of the 1993 International Olympic Committee's President's Prize for my work in the field of doping.

I have served as an expert on anabolic steroids to the United States Department of Justice, and since 1999, I have advised the Office of National Drug Control Policy on matters of doping.

I was intimately involved in the formation of the World Anti-Doping Agency, served on a number of its committees, including its Health, Medicine and Research Committee, its Therapeutic Use Exemption Committee, and its Athlete's Passport Committee. Currently, I represent the United States as a member of the WADA's Prohibited List and Methods Committee where, on occasion, I have served as Acting Chair.

I have no vested interest in testifying today other than to share my views with the Committee about the complex issue of Major League Baseball and the use of performance-enhancing drugs.

Since appearing before the United States Senate Committee on Commerce, Science and transportation in 1999 to discuss the use of performance-enhancing drugs in Olympic competition, there has been a sea change on many fronts.

At the federal level, we have witnessed great strides both nationally and internationally in the fight against doping.

The President highlighted the issues in his 2004 State of the Union, the Department of Justice has pursued the BALCO investigation, and the FDA removed ephedra and androstenedione from the store shelf.

Just last month, the recently enacted Anabolic Steroid Control Act of 2004 became effective adding numerous so-called steroid precursors to the list of anabolic steroids controlled under the federal Controlled Substances Act. This Act increased the government's commitment to education regarding the dangers of anabolic steroids while requiring a review of the federal sentencing guidelines for criminal offenses involving anabolic steroids.

The United States Government has also demonstrated its commitment through increased funding of several anti-doping initiatives. For example, just last year, \$7.5 million was appropriated to support the United States Anti-Doping Agency's (USADA) testing research and education programs. It was USADA that played a critical role in the BALCO revelations and in unmasking the numerous issues associated with the designer steroid, THG.

Internationally, the United States, with the Office on National Drug Control Policy at the helm, has played a leadership role in both the formation of WADA and in its ongoing governance and funding.

Specifically, in 2004, the United States Government contributed a historic and unprecedented \$1.45 million towards WADA's \$23 million budget (60% to research, 15% to out-of-competition testing, 15% to education, 10% for contingency) and that unprecedented level of commitment continues as reflected in the President's fiscal year 2006 budget.

WADA was created in November 1999 to support and promote fundamental values in sport. WADA was set up as a foundation under the initiative of the IOC with the support and participation of intergovernmental organizations, governments, public authorities and other public and private bodies fighting against doping in sport. The agency has equal representation from the Olympic Movement and from public authorities.

The United States is one of only five nations serving on WADA's Executive Committee. It chaired WADA's Ethics and Education Committee, and most recently, the United States Government has assumed a strong leadership position during the drafting of the anti-doping convention under the auspices of UNESCO.

With this as a backdrop, one must ask the question where have we gone astray with Major League Baseball and why should we care?

These questions can be addressed from many perspectives.

Perhaps the seminal moment in surfacing the current issue of performance-enhancing drug use baseball was in 1998 with the revelation that Mark McGwire had used androstenedione during his record-breaking 70 home run season. At the time, McGwire's use did not violate the laws of the land, nor the laws of baseball – both were to change.

The 2002 assertions of Jose Conseco and the late Ken Caminiti that steroid abuse was rampant in organized baseball were dismissed by many in baseball as being hyperbolic. However, last week Mr. Selig acknowledged that in 2001, that in fact, 11 percent of Minor League players had tested positive, and baseball's own 2003 "Survey Testing" had revealed that even with a very porous testing program, as many as 5 to 7 percent of Major League players had tested positive – the equivalent of two major league rosters.

Two weeks ago, we learned that in 2004, though employing a porous testing program, 1% to 2% tested positive, which still translates to an unacceptable numbers of users - between 12 and 24 league wide, the equivalent of a team to a team roster. The incidence would likely have been higher if the testing had been performed, as it should have been - year round, in and out of competition, random, no notice basis.

The incidence would likely have been higher if the testing had been performed, as it should have been - year round, in and out of competition, random, on a no notice basis.

To put these figures in perspective, compare Major League Baseball's statistics with those of WADA, where ½ percent of the 150,000 tests rigorously administered worldwide in 2003 were positive for steroids.

One can only conclude that the prior assertions of rampant steroid abuse in baseball were likely not hyperbolic.

Why should we care?

We should care for many reasons, but perhaps most notable, is that baseball, our national pastime, for better or for worse is a role model sport and likely contributes to the alarming abuse of anabolic steroids by teenagers. Just reflect on the enormous increase in sales of androstenedione (andro), the year after Mark McGwire broke Roger Maris' long-standing home run record.

The most recent data from the annual National Institute of Drug Abuse's Monitoring the Future survey reveals that in 2004, 3.4% of 12th graders have used these drugs at some time in their lifetime and as many as 1.9% of 8th graders have used them – very disturbing statistics.

And even more alarming is the perception amongst high school students that they are harmful has dropped significantly from 71% in 1992 to 56% in 2004.

And let me assure you from a public health perspective the abuse of these drugs is harmful both physically and behaviorally. Their abuse can lead to an array of physical problems, even with therapeutically prescribed doses, some predictable, some not, some permanent and some not.

Some adverse effects are visible to the naked eye, while others are not. If anabolic steroids are injected, the transmission of HIV and hepatitis B through shared needles and vials use is a very real concern.

Additionally, unlike almost all other drugs, the adverse effects of steroid based hormones share a unique characteristic -- their dangers may not be manifest for months, years and even decades.

With regard to physical side effects in males, their use may result in feminization with symptoms such as breast development, high-pitched voice, testicular atrophy, and impotency. This is because anabolic steroids may be converted in the body to estrogens in a process known as aromatization. The abuse of these drugs by women may result in their masculinization.

Both sexes can experience the following effects, which range from the merely unsightly to the life endangering. They include severe acne, bloating and rapid weight gain, clotting disorders, liver damage, premature heart disease and stroke, elevated total cholesterol and LDL levels with depressed HDL levels and increased tendinous injuries.

In adolescents, anabolic steroids can result in the premature closure of the epiphyses (growth centers in bone), such that the adolescent will never reach their genetically determined height.

The abuse of anabolic steroids can cause severe mood swings with marked irritability, depression, and with feelings of invincibility. Antisocial behaviors may be manifest by bouts of outright aggression commonly referred to as "roid rage".

Regular use of anabolic steroids can result in a dependency syndrome, which can result in the development of a profound depression that can lead to suicide.

But baseball's problem is not limited to steroids.

One can only wonder why baseball's new drug policy does not explicitly ban amphetamines.

It was amphetamine abuse that gave rise to both the controlled substances act of 1970, and to the development of the Olympic banned substances list in 1968, following the first recorded fatalities from performance-enhancing drugs, namely, amphetamines.

Amphetamines, which are stimulants, have an array of adverse effects associated with their use and abuse.

Acute side effects include: increased heart rate, increased blood pressure, reduced appetite and weight loss, insomnia, headaches, convulsions, hallucinations and paranoia, and death may also occur due to cerebral hemorrhages, heart attacks, heart rhythm abnormalities and heatstroke. Chronic side effects include: uncontrollable and abnormal movements of the face and jaw muscles called dyskinesias, compulsive and repetitive behaviors, paranoid delusions, systemic vascular disorders and nerve damage.

While ephedra is now banned in baseball subsequent to the heatstroke death of Steven Bechler and its being banned by the FDA, one should not lose sight of the fact that ephedra is closely related to the stimulant, amphetamine.

Why ephedra is banned by MLB and amphetamine is not remains an enigma.

The position that the player's association has taken with respect to amphetamines certainly leads one to suspect that they too are endemic in baseball.

Finally, a few words about MLB's new drug testing policy.

In my judgment, the policy as best we know it, can best be described as one of incrementalism - one designed to silence its critics, but one not designed to seriously rid professional baseball of the abuse of all performance-enhancing drugs.

And to be sure the devil is in the details. For example, while human growth hormone is on baseball's banned list, baseball will not conduct blood testing which is the only way it can currently be detected.

Doping is an exquisitely complex subject involving the interplay of numerous disciplines - chemistry, physiology, pharmacology, laboratory science, therapeutics and therapeutic exemptions, results management including sanctions, law, and least but not least, athlete's rights.

The interplay of these disciplines, as detailed in the International Standards of the World Anti-Doping Agency, has been approved by sporting bodies and governments worldwide. These Standards provide the blueprints and guideposts that are essential to an effective, transparent, and accountable anti-doping program.

In my opinion, the complexity of anti-doping far exceeds the capacity of baseball to design, implement and monitor an effective, transparent and accountable program.

It is beyond the scope of two attorneys and two physicians, one each from MLB and from the MLB Player's Association as called for in the 2003 agreement between the two entities.

It is noteworthy that the gold standard for anti-doping already exists.

It is embodied in the World Anti-Doping Code and its International Standards and Major League Baseball should embrace them.

Major League Baseball should heed the experience of the Olympic Movement, which recognized that its credibility, its very essence, was cracking under the weight of doping and so it passed the anti-doping baton to WADA and to national anti-doping agencies, such as USADA.

I am pleased to note that baseball has taken one significant step in that direction by contracting out its anti-doping laboratory services to a WADA accredited laboratory.

At a minimum, and now I am being very specific, as a next step, Major League Baseball should adopt the WADA List of Prohibited Substance and Methods in its entirety, as well as its existing testing protocols.

The List of Prohibited Substances and Methods is a continuously evolving product of countless man-hours experts, scientists and physicians from around the world. It is endorsed by sporting bodies, world wide, as well as by the governments of the world, including the United States.

While the potential of a two-year sanction for steroid abuse, as called for in the World Anti-Doping Code, may make baseball hesitant to embrace the Code, Major League Baseball should be aware that the Code calls for sanctions to be reduced in "exceptional circumstances", and provides for the possible reduction or elimination of the period of ineligibility in the unique circumstances where the athlete can establish that he had no fault or negligence in connection with the violation.

By adopting the World Anti-Doping Code, Major League Baseball would not be alone in so doing as a high profile professional sport. For example, currently men's professional tennis (ATP), soccer (FIFA), and professional cycling (UCI) are signatories to the Code.

Furthermore, United States Anti-Doping Agency, USADA, is in the best position to implement the best practices of doping control in Major League Baseball in conformity with the requirements set forth in the World Anti-Doping Code.

Finally, only when baseball demonstrates its unabashed commitment to drug free sport will it fully regain the confidence of its fans and once again deservedly become America's favorite pastime.

Websites: www.wada-ama.org (World Anti-Doping Agency) (WADA)
www.wada-ama.org/rtecontent/document/list_book_2005_en.pdf
(The 2005 Prohibited List)
www.usada.org (United States Anti-Doping Agency) (USADA)

**ESPN.com:** Cycling[\[Print without images\]](#)

Thursday, July 27, 2006

WADA panel member Wadler Q&A

ESPN.com

Dr. Gary Wadler, a member of the World Anti-Doping Agency's prohibited list and methods committee, spoke Thursday with ESPN.com about Floyd Landis, testosterone and testing.

Question: What do today's events mean for Floyd Landis and his victory in the Tour de France?

Answer: I would have been a lot happier if this had not appeared in the public domain until there was a confirmation of the "B" sample. Typically, the "B" sample confirms the "A" sample, but you don't know that until you do it. ... What we're hearing now is conjecture. If it is confirmed, it would be another very sad day in the long history of elite cycling.

Q: How often does a "B" sample differ from an "A" sample?

A: It's extraordinarily unusual. It's not common. The technology is quite sophisticated these days and you don't find that occurring very often. I'd be surprised if the "B" specimen doesn't confirm the "A," but until we know, we don't know. We have to be fair to athletes. They have due process. They are innocent until proven guilty. We should not be trying [anyone] until we know what the findings were, and he will have a chance to appeal this. We have to be a little bit cautious.

Q: Under World Anti-Doping Agency regulations, what is an acceptable testosterone-to-epitestosterone ratio?

A: It used to be 6-to-1. It's now 4-to-1. Most people are 1-to-1 or 2-to-1.

Q: Does a test positive for high levels of testosterone equate to a failed test?

A: The question for any given individual is, what does an abnormal or elevated ratio mean? Some people are born with [ratios] that are normally 4-to-1, or maybe 8-to-1. If they are consistently that way, week after week, there are ways to evaluate that. If the elevation exists and occurs naturally in that athlete, we can figure that out. This particular athlete -- Landis, in this case -- I'm sure has had multiple drug tests and a history of his testosterone to epitestosterone ratios, and if there's a sudden aberration, it sends up a red flag.

Q: For a cyclist, what's the benefit of elevated levels of testosterone? Why would a cyclist use it?

A: It's certainly not one of the first-line drugs one thinks of for racing. Steroids can increase strength and improve recovery time and prevent the breakdown of muscle, maybe make him more assertive and aggressive. All of those could have some positive attribute. But most steroids are given in cycles [6-12 weeks] and in context of working out in a gym with weights. It makes no sense to me why an athlete would take testosterone the day of a race when it doesn't work that way. It doesn't make sense in terms of the pharmacology of the drug, and it really doesn't have the attributes that would be attractive to a cyclist -- particularly one running the risk of violating anti-doping regulations.

Everybody knew the spotlight was on cycling. For eight years, the world has been watching cycling particularly closely. It would be the ultimate form of denial, or the ultimate sense of invincibility, to think you're going to evade that. And when the pharmacology of the drug doesn't really, in my judgment, seem like a drug of particular note to a cyclist, it doesn't really compute.

GDC0062

Q: When should we expect to learn the results of the "B" sample?

A: Certainly, with all that's going on, you could expect to see results within 24-48 hours.

What worries me is we will succumb to doping fatigue, that we will turn off our interest in doping, particularly the impact it has on young people. So much of our sports culture has doping issues around it, and people are getting tired of it. And they can't afford to get tired of it. The price is far too great for our society to develop a laissez-faire attitude. We cannot succumb to doping fatigue.

ESPN.com: [Help](#) | [PR Media Kit](#) | [Sales Media Kit](#) | [Report a Bug](#) | [Contact Us](#) | [Site Map](#) | [Jobs at ESPN](#) | **Supplier Information** | Copyright ©2007 ESPN Internet Ventures. [Terms of Use](#) and [Privacy Policy](#) and [Safety Information/Your California Privacy Rights](#) are applicable to you. All rights reserved.

GDC0063

PART 14 ANTI-DOPING RULES OF THE UCI

(new rules entered into force on 13 August 2004)

INTRODUCTION

Pursuant to amendments adopted by the 115th session of the International Olympic Committee in July 2003, the Olympic Charter stipulates that in order to be recognized by the IOC, an International Federation must adopt and implement the World Anti-Doping Code (Rule 29).

To be eligible for a participation in the Olympic Games, a competitor, coach, trainer or official must respect and comply in all aspects with the World Anti-Doping Code (Rule 45).

As a consequence, at its meeting of 22-23 July 2004, the UCI Management Committee decided to accept the World Anti-Doping Code and to incorporate the Code in UCI's Regulations, as is done in these Anti-Doping Rules.

Terms in italiques are defined in appendix 1.

ANTI-DOPING RULES OF THE UCI

SUMMARY

| | Page |
|---|------|
| Chapter I SCOPE | 1 |
| Chapter II DOPING | 3 |
| Chapter III THE <i>PROHIBITED LIST</i> | 6 |
| Chapter IV THERAPEUTIC USE EXEMPTION | 7 |
| Chapter V WHEREABOUTS INFORMATION | 13 |
| Chapter VI TESTING | 16 |
| Chapter VII RESULTS MANAGEMENT | 25 |
| Chapter VIII PROVISIONAL MEASURES | 30 |
| Chapter IX RIGHT TO A FAIR HEARING | 31 |
| Chapter X SANCTIONS AND CONSEQUENCES | 35 |
| Chapter XI APPEAL TO THE CAS | 41 |
| Chapter XII CONFIDENTIALITY AND PUBLIC DISCLOSURE | 42 |
| Chapter XIII FINAL PROVISIONS | 44 |
| Appendix 1: DEFINITIONS | 51 |
| Appendix 2: SELECTION OF RIDERS TO BE TESTED | 53 |
| Appendix 3: SELECTION OF RIDERS TO BE TESTED | 55 |
| Appendix 4: PLAN OF STANDARD TESTING STATION FOR ANTIDOPING TESTS | 59 |
| Appendix 5: EQUIPMENT OF THE DOPING CONTROL STATION | 60 |
| Appendix 6: ANTIDOPING CONTROL | 61 |
| Appendix 7: NOTIFICATION TO THE RIDER | 62 |
| Appendix 8: NO-SHOW REPORT | 63 |
| Appendix 9: NOTIFICATION TO THE RIDER OF A POSITIVE RESULT | 64 |
| Appendix 10: REQUEST FOR COUNTER-ANALYSIS | 65 |
| Appendix 11: LIST OF MEDICINES TAKEN | 66 |
| Appendix 12: CODE OF SPORTS-RELATED ARBITRATION (CAS) | 67 |

Chapter SCOPE

- 1.** These Anti-Doping Rules shall apply to all *License-Holders*.

In-Competition Testing

- 2.** *Riders* participating in *International Events* shall be subject to *In-Competition Testing* under these Anti-Doping Rules.

Riders participating in *National Events* shall be subject to *In-Competition Testing* initiated and directed by the *National Anti-Doping Organization* of the country or any other organization or person so authorized by that *National Anti-Doping Organization*. *Doping Control* shall be governed by the anti-doping rules of the *National Anti-Doping Organization*.

Comment: As results from the above article, these Anti-Doping Rules do not apply at *National Events*.

- 3.** *In-Competition Testing* at *International Events* may be initiated and directed by the UCI or by the National Federation of the country or any other organization or person so authorized by the UCI. *Doping Control* shall be governed by these Anti-Doping Rules exclusively.

- 4.** If the UCI decides not to conduct any *Testing* at an *International Event*, the *National Anti-Doping Organization* for the country where the *Event* takes place, may initiate and conduct such *Testing* in coordination with and with the approval of the UCI or, if the UCI withholds its approval, with the approval of *WADA*. In such cases, *Doping Control* shall be governed by the anti-doping rules of that *National Anti-Doping Organization*.

- 5.** *Riders* shall be subject to *In-Competition Testing* at the Olympic Games, the Paralympic Games and events of *Major Event Organizations*.

Doping Control shall be governed by the rules of the International Olympic Committee, the International Paralympic Committee and the *Major Event Organizations* respectively. However, results management and the conduct of hearings shall be referred to the UCI as far as sanctions beyond *Disqualifications* from the event or the results of the event.

Out-of-Competition Testing

- 6.** *Riders* shall be subject to *Out-of-Competition Testing*.
- 7.** *Out-of-Competition Testing* may be initiated and conducted by the UCI or by the National Federation of the country or any other organization or person so authorized by the UCI.

UCI CYCLING REGULATIONS

Doping Control shall be governed by these Anti-Doping Rules exclusively.

8. Riders shall also be subject to *Out-of-Competition Testing* initiated and conducted by any other *Anti-Doping Organization* that is so authorized under the *Code*:
1. WADA;
 2. The International Olympic Committee or the International Paralympic Committee in connection with the Olympic Games or Paralympic Games;
 3. The *National Anti-Doping Organization* of the Rider;
 4. The *National Anti-Doping Organization* of any country where the Rider is present.

Doping Control shall be governed by the anti-doping rules of the *Anti-Doping Organization* concerned.

However, results management and the conduct of hearings from a test by the International Olympic Committee or the International Paralympic Committee shall be referred to the UCI as far as sanctions beyond *Disqualification* from the event or the results of the event.

- Comment:
- 1) As is expressed in the above article, any Rider may be tested *Out-of-Competition* by any of the above mentioned organizations, according to the rules of the organization conducting the test.
 - 2) National Federations may not initiate and conduct *Out-of-Competition* testing, including on their national level Riders, unless so authorized by the UCI or another *Anti-Doping Organization*.

Anti-doping violations where no *Sample* collection is involved

9. The UCI has jurisdiction for and these Anti-Doping Rules shall apply to any anti-doping violation committed by a *License-Holder* where no *Sample* collection is involved and that is discovered:
- (i) by the UCI, by one of its constituents or member Federations, by one of their officials, officers, staff members, members, *License-Holders*, or any other body or individual that is subject to the regulations of the UCI or one of its member Federations; or
 - (ii) by a body or individual that is not an *Anti-Doping Organization*.
10. If an anti-doping violation where no *Sample* collection is involved is discovered by another *Anti-Doping Organization*, the anti-doping rules of that *Anti-Doping Organization* shall apply.

However, if the violation is discovered by the International Olympic Committee or the International Paralympic Committee, results management and the conduct of hearings shall be referred to the UCI as far as sanctions beyond *Disqualification* from the event or the results of the event.

Results management concerning foreign or non-resident Riders

- 11.** Results management and the conduct of hearings for an anti-doping rule violation arising from a test by, or discovered by, a *National Anti-Doping Organization* involving a *License-Holder* that is not a citizen or resident of that country shall be administered by and under the rules of that *National Anti-Doping Organization*.

Unauthorized Testing

- 12.** If a *Rider* refuses a test by an *Anti-Doping Organization* that has no authority to test under these Anti-Doping Rules or under the *Code*, such refusal shall not constitute an anti-doping violation under these Anti-Doping Rules.
- 13.** If a *Rider* has been tested by an *Anti-Doping Organization* that has no authority to test under these Anti-Doping Rules or under the *Code* and the test results in an *Adverse Analytical Finding*, the UCI shall have jurisdiction and these Anti-Doping Rules shall apply.

General comment:

- 1) Under the *Code*, *National Federations* have no jurisdiction in *Doping Control*.

However, Anti-Doping Organizations having jurisdiction under the Code, may delegate jurisdiction to National Federations.

The involvement of National Federations in doping control at the international level is laid down in these Anti-Doping Rules.

National Federations and their respective National Anti-Doping Organization may agree on the Federation's involvement in Doping Control at the national level.

- 2) In addition to the obligations to submit to Testing pursuant to these Anti-Doping Rules and the *Code*, *Riders* may also be obliged to submit to Testing and be sanctioned for anti-doping violations pursuant to local anti-doping law.

Chapter DOPING

Definition of doping

- 14.** Doping is defined as the occurrence of one or more of the anti-doping rule violations set forth in article 15.

Anti-doping rule violations

15. The following constitute anti-doping rule violations:

1. The presence of a *Prohibited Substance* or its *Metabolites* or *Markers* in a *Rider's* bodily *Specimen*.

- 1.1. It is each *Rider's* personal duty to ensure that no *Prohibited Substance* enters his body. *Riders* are responsible for any *Prohibited Substance* or its *Metabolites* or *Markers* found to be present in their bodily *Specimens*. Accordingly, it is not necessary that intent, fault, negligence or knowing *Use* on the *Rider's* part be demonstrated in order to establish an anti-doping violation under article 15.1.

Warning:

- 1) *Riders must refrain from using any substance, foodstuff, food supplement or drink of which they do not know the composition. It must be emphasized that the composition indicated on a product is not always complete. The product may contain Prohibited Substances not listed in the composition.*
- 2) *Medical treatment is no excuse for using Prohibited Substances or Prohibited Methods, except where the rules governing Therapeutic Use Exemptions are complied with.*

- 1.2. Excepting those substances for which a threshold concentration is specifically identified in the *Prohibited List*, the detected presence of any quantity of a *Prohibited Substance* or its *Metabolites* or *Markers* in a *Rider's Sample* shall constitute an anti-doping rule violation.

- 1.3. As an exception to the general rule of article 15.1, the *Prohibited List* may establish special criteria for the evaluation of *Prohibited Substances* that can also be produced endogenously.

2. *Use or Attempted Use of a Prohibited Substance or a Prohibited Method.*

- 2.1. The success or failure of the *Use of a Prohibited Substance or Prohibited Method* is not material. It is sufficient that the *Prohibited Substance or Prohibited Method* was *Used or Attempted* to be *Used* for an anti-doping rule violation to be committed.

3. *Evading Sample collection or, after notification as authorized under these Anti-Doping Rules, refusing, or failing without compelling justification, to submit to Sample collection or, regarding the Riders referred to in article 122, to check in for Sample collection.*

4. *Violation of the requirements regarding Rider availability for Out-of-Competition Testing including failure to provide required whereabouts information and missed tests as set forth in article 86.*

5. *Tampering, or Attempting to tamper, with any part of Doping Control.*

6. *Possession of Prohibited Substances and Methods.*

- 6.1 Possession by a Rider at any time or place of a *Prohibited Substance* or a *Prohibited Method* referred to in article 15.6.3 below, unless the Rider establishes that the Possession is pursuant to a Therapeutic Use Exemption granted in accordance with chapter IV or other acceptable justification.
- 6.2 Possession of a *Prohibited Substance* or a *Prohibited Method* referred to in article 15.6.3 below, by Rider's Support Personnel in connection with a Rider, Event or training, unless the Rider's Support Personnel establishes that the Possession is pursuant to a therapeutic use exemption granted to a Rider in accordance with chapter IV or other acceptable justification.
- 6.3 In relation to Possession, the following categories of substances and methods of the *Prohibited List* are prohibited:

Categories of Prohibited Substances:

- S1. Anabolic agents
- S2. Hormones and related substances
- S3. Beta-2 agonists
- S4. Agents with anti-oestrogenic activity
- S5. Diuretics and other masking agents

Categories of Prohibited Methods:

- M1. Enhancement of oxygen transfer
- M2. Chemical and physical manipulation
- M3. Gene doping

7. Trafficking in any *Prohibited Substance* or *Prohibited Method*.
8. Administration or Attempted administration of a *Prohibited Substance* or *Prohibited Method* to any Rider, or assisting, encouraging, aiding, abetting, covering up or any other type of complicity involving an anti-doping rule violation or any Attempted violation.

Proof of doping

Burdens and standards of proof

16. The UCI and its National Federations shall have the burden of establishing that an anti-doping rule violation has occurred. The standard of proof shall be whether the UCI or its National Federation has established an anti-doping rule violation to the comfortable satisfaction of the hearing body bearing in mind the seriousness of the allegation which is made. This standard of proof in all cases is greater than a mere balance of probability but less than proof beyond a reasonable doubt. Where these Anti-Doping Rules place the burden of proof upon the Rider or other Person alleged to have committed an anti-doping rule violation to rebut a presumption or establish specified facts or circumstances, the standard of proof shall be by a balance of probability.

Methods of establishing facts and presumptions

- 17.** Facts related to anti-doping rule violations may be established by any reliable means, including admissions.
- 18.** WADA-accredited laboratories or as otherwise approved by WADA are presumed to have conducted *Sample* analysis and custodial procedures in accordance with the *International Standard* for laboratory analysis. The *Rider* may rebut this presumption by establishing that a departure from the *International Standard* occurred.

If the *Rider* rebuts the preceding presumption by showing that a departure from the *International Standard* occurred, then the UCI or the National Federation shall have the burden to establish that such departure did not cause the *Adverse Analytical Finding*.

- 19.** Departures from these Anti-Doping Rules, the *Procedural Guidelines* set by the Anti-Doping Commission or the *International Standard for Testing* which did not cause an *Adverse Analytical Finding* or other anti-doping rule violation shall not invalidate such findings or results. If the *Rider* establishes that departures from these Anti-Doping Rules, the *Procedural Guidelines* or the *International Standard* occurred during *Testing* then the UCI or its National Federation shall have the burden to establish that such departures did not cause the *Adverse Analytical Finding* or the factual basis for the anti-doping rule violation.
- 20.** Any *Anti-Doping Inspector*, *Medical Inspector*, commissaire or official shall draw up a detailed report of any anti-doping rule violation and of any incident, anomaly or irregularity concerning *Testing* which he may observe or which may be reported to him. He shall note the identity of any witnesses. Witness statements may be included in the report and countersigned by the witnesses. This report and all the supporting documentation must be sent without delay to the UCI Anti-Doping Commission.



Chapter THE PROHIBITED LIST

- 21.** These Anti-Doping Rules incorporate the *Prohibited List* which is published and revised by WADA as described in article 4.1 of the *Code*. The UCI will publish the current *Prohibited List* also in UCI's Official News Bulletin.

Comment:

- 1) the *Prohibited List* currently in force may also be found on UCI's website at www.uci.ch.
- 2) Most sections of the *Prohibited List* refer to categories of *Prohibited Substances* or *Prohibited Methods*, while only a limited number of these *Substances* or *Methods* are listed under that category; yet other *Substances* or *Methods* than those recited are prohibited as is indicated for the respective categories.

- 3) *The Prohibited List relates to a sports regulation. The Use or Possession of, and Traffic in, a number of substances on the List is also prohibited or regulated in the national laws of many countries. Criminal sanctions may apply. A substance or method that is not prohibited under the List may be prohibited or regulated under national law.*
22. Unless provided otherwise in the *Prohibited List* and/or a revision, the *Prohibited List* and revisions shall go into effect under these Anti-Doping Rules 3 (three) months after publication of the *Prohibited List* by WADA on WADA's website at www.wada-ama.org, without requiring any further action by the UCI.
23. WADA's determination of the *Prohibited Substances* and *Prohibited Methods* that are or will be included on the *Prohibited List* shall be final and not subject to challenge by a *License-Holder*.

IV

Chapter THERAPEUTIC USE EXEMPTION (TUE)

24. *Riders with a documented medical condition requiring the Use of a Prohibited Substance or a Prohibited Method must, prior to their participation in any International Event, obtain a Therapeutic Use Exemption (TUE) from the UCI, regardless of whether the Rider previously has received a TUE from his National Anti-Doping Organization.*

However, Riders of the juniors and masters categories shall obtain a TUE from their National Anti-Doping Organization or such authority as appointed by their National Anti-Doping Organization.

25. *Riders with a documented medical condition requiring the Use of a Prohibited Substance or a Prohibited Method who are not planning to participate in an International Event must, prior to their participation in any National Event, obtain a Therapeutic Use Exemption from their National Anti-Doping Organization or such authority as appointed by their National Anti-Doping Organization. The procedure for granting such TUE is governed by the rules of that National Anti-Doping Organization.*
26. The following articles of this chapter IV govern TUE's requested from the UCI.
27. A Therapeutic Use Exemption is granted according to a standard procedure (articles 38 - 45). For the *Prohibited Substances* or *Prohibited Methods* referred to in article 47, an abbreviated procedure is also available (articles 46 - 50).
28. Decisions regarding the grant, withdrawal and denial of TUE's are taken in the first instance by the Therapeutic Use Exemption Committee of the UCI.

Therapeutic Use Exemption Committee (TUEC)

29. The UCI shall appoint a committee of at least 3 (three) physicians to consider requests for TUE's: the Therapeutic Use Exemption Committee (TUEC).
30. The members of the TUEC, or at least 3 (three) of them, shall be physicians with experience in the care and treatment of athletes and a sound knowledge of clinical, sports and exercise medicine.
31. A majority of the members of the TUEC should not have any official responsibility in the UCI or a National Federation. All members of the TUEC will sign a conflict of interest agreement.
32. The TUEC may seek whatever medical or scientific expertise it deems appropriate in reviewing the circumstances of any application for a TUE.
33. In applications involving *Riders* with disabilities, advice shall be sought from an expert possessing specific experience with the care and treatment of athletes with disabilities, if no member of the TUEC possesses such experience.
34. Upon the UCI's receipt of a TUE application, the Chair of the TUEC shall appoint one or more members of the TUEC (which may include the Chair) to consider such application and render a decision promptly.

Criteria for granting a Therapeutic Use Exemption

35. A Therapeutic Use Exemption will be granted only in strict accordance with the following criteria:
 1. The *Rider* should submit an application for a TUE on a form provided by the UCI no less than 21 (twenty-one) days before participating in an *Event*.
 2. The *Rider* would experience a significant impairment to health if the *Prohibited Substance* or *Prohibited Method* were to be withheld in the course of treating an acute or chronic medical condition.
 3. The therapeutic use of the *Prohibited Substance* or *Prohibited Method* would produce no additional enhancement of performance other than that which might be anticipated by a return to a state of normal health following the treatment of a legitimate medical condition. The *Use* of any *Prohibited Substance* or *Prohibited Method* to increase "low-normal" levels of any endogenous hormone is not considered an acceptable therapeutic intervention.
 4. There is no reasonable therapeutic alternative to the *Use* of the otherwise *Prohibited Substance* or *Prohibited Method*.
 5. The necessity for the use of the otherwise *Prohibited Substance* or *Prohibited Method* cannot be a consequence, wholly or in part, of prior non-therapeutic *Use* of any *Prohibited Substance* or *Prohibited Method*.
 6. An application for a TUE will not be considered for retroactive approval except in cases where:
 - a. Emergency treatment or treatment of an acute medical condition was necessary, or
 - b. Due to exceptional circumstances, there was insufficient time or opportunity for a *Rider* to submit an application 21 (twenty-one) days prior to his participation in any *Event*, or the TUEC to consider the application prior to the *Rider's* participation in any *Event*.

UCI CYCLING REGULATIONS

- 36.** A TUE shall be denied on the ground of the impossibility or difficulty to control the dose, frequency, route of administration or any other aspect of the *Use of a Prohibited Substance or Prohibited Method*, that may produce enhancement of performance other than that allowed under article 35.3.
- 37.** The TUEC may submit the granting of a TUE to any conditions that it may specify.

Standard Therapeutic Use Exemption procedure

- 38.** A TUE will only be considered following the receipt of:
- (i) a legibly completed application form that complies with these Anti-Doping Rules and that must include all relevant information and documents, and
 - (ii) an application fee, the amount of which shall be set annually by the Management Committee.

Warning: *Any file that is not complete or not legible will not be considered as valid and will be returned to the sender.*

- 39.** The TUE application form(s), as set out in the *International Standard for Therapeutic Use Exemptions*, can be modified by the UCI Anti-Doping Commission to include additional requests for information, but no sections or items shall be removed.

- 40.** The TUE application form(s) shall be completed legibly, in English or French. Any information attached to the TUE application form shall be in English or French. If such information has been originally established in another language, the *Rider* shall attach the original document and a translation in English or French.

- 41.** A *Rider* may not apply to more than one *Anti-Doping Organization* for a TUE. The application must list any previous and/or current requests for permission to use an otherwise *Prohibited Substance* or *Prohibited Method*, the body to whom that request was made, and the decision of that body.

- 42.** The application must include a comprehensive medical history and the results of all examinations, laboratory investigations and imaging studies relevant to the application.

Any additional relevant investigations, examinations or imaging studies requested by the TUEC will be undertaken at the expense of the Rider.

- 43.** The application must include a statement by an appropriately qualified physician attesting to the necessity of the otherwise *Prohibited Substance* or *Prohibited Method* in the treatment of the *Rider* and describing why an alternative, permitted medication cannot, or could not, be used in the treatment of this condition.

The dose, frequency, route and duration of administration of the otherwise Prohibited Substance or Prohibited Method in question must be specified.

- 44.** The decision of the TUEC will be conveyed in writing to the *Rider* by the Anti-Doping Commission.

UCI CYCLING REGULATIONS

- 45.** Where a TUE has been granted, the *Rider* and *WADA* will be promptly provided with a certificate of approval for therapeutic use which includes information pertaining to the duration of the exemption and any conditions associated with the TUE. *WADA* will also be provided with all supporting documentation.

The *Rider* shall carry a copy of the certificate with him at all times and present it to the *Anti-Doping Inspector* at the time of *Testing*.

Abbreviated Therapeutic Use Exemption procedure

- 46.** It is acknowledged that some substances included on the *Prohibited List* are used to treat medical conditions frequently encountered in the athlete population. In such cases, a full application as detailed in article 35.4, and articles 38 - 45, is unnecessary. Accordingly an abbreviated process of the TUE is established.
- 47.** The *Prohibited Substances* or *Prohibited Methods* which may be permitted by this abbreviated process are strictly limited to the following: beta-2 agonists (formoterol, salbutamol, salmeterol and terbutaline) by inhalation and glucocorticosteroids by non-systemic routes.
- 48.** Approval for Use of one of the *Prohibited Substances* referred to in article 47 is effective upon receipt of a complete notification by the UCI.

The diagnosis and when applicable any tests undertaken in order to establish that diagnosis should be included (without the actual results or details).

Articles 39, 40 and 43 apply.

Notifications that are incomplete, illegible or do not comply with these Anti-Doping Rules, shall carry no exemption and shall be returned to the applicant.

- 49.** The UCI will advise *WADA* and the *Rider's* National Federation and *National Anti-Doping Organization* on receipt of a regular notification.

The UCI will provide *WADA* with the application for the TUE.

- 50.** A notification for a TUE will not be considered for retroactive approval except in cases where:
- emergency treatment or treatment of an acute medical condition was necessary, or
 - due to exceptional circumstances, there was insufficient time or opportunity for a *Rider* to submit, or the UCI to receive, the application prior to the *Rider's* participation in any *Event*.

Confidentiality of information

51. By applying for a TUE, the *Rider* provides consent for the transmission of all information pertaining to the application to members of the TUEC of the UCI and the TUEC of WADA and, as required, other independent medical or scientific experts, or to all necessary staff involved in the management, review or appeal of TUEs and for the decisions of the TUECs to be distributed to other relevant *Anti-Doping Organizations* under the provisions of the *Code*.
52. Should the *Rider* wish to revoke the right of the TUEC of the UCI or the TUEC of WADA to obtain any health information on his behalf, the *Rider* must notify his medical practitioners in writing of the fact. As a consequence of such a decision, the *Rider* will not receive approval for a TUE or renewal of an existing TUE.
53. Should the assistance of external, independent experts be required, all details of the application will be circulated without identifying the *Rider* and the medical practitioners involved in the *Rider's* care.
54. The members of the TUECs and the administration of the *Anti-Doping Organizations* involved will conduct all of their activities in strict confidence.

Duration of TUE

55. Each TUE will have a specified duration as decided upon by the TUEC. The duration shall be indicated in the certificate of approval for therapeutic use.

No TUE may have a duration of more than 1 (one) year. It may be renewed.

The TUE expires at the end of its duration.

Withdrawal of TUE

TUE granted under standard procedure

56. The TUEC of the UCI may review and withdraw the TUE at any time during the duration of the TUE.
57. The decision of the TUEC of the UCI withdrawing the TUE and the decision of WADA or the CAS reversing the granting of the TUE, shall take effect 14 (fourteen) days following notification of the decision to the *Rider*, unless the decision sets a shorter term.

TUE granted under abbreviated procedure

58. The TUEC of the UCI and the TUEC of WADA may review and withdraw the TUE at any time during the duration of the TUE.
59. A decision of the TUEC of the UCI or the TUEC of WADA withdrawing the TUE and a decision of the CAS reversing the granting of the TUE, shall take effect immediately following notification of the decision to the *Rider*.

The *Rider* will nevertheless be able to apply for a TUE in accordance with the standard procedure.

Results before withdrawal

- 60.** The withdrawal of a TUE and the decision reversing the granting of a TUE shall not apply retroactively. They shall not disqualify the *Rider's* results before the moment that the decision takes effect.

Results after expiry or withdrawal

- 61.** The Anti-Doping Commission, when conducting the initial review of an *Adverse Analytical Finding*, will consider whether the finding is consistent with expiry or withdrawal of the TUE.

Information

- 62.** The *Rider* and all relevant *Anti-Doping Organizations* shall be notified immediately of any decision withdrawing a TUE or reversing the granting of a TUE.

Review by WADA and appeal to the CAS

Review by WADA at the request of the Rider

- 63.** The *Rider* may request *WADA* to reverse the decision by which the TUEC of the UCI denies or withdraws a TUE.
- 64.** The *Rider* shall provide to the TUEC of *WADA* all the information for a TUE as submitted initially to the UCI, accompanied by the application fee that *WADA* may request. The TUEC of *WADA* will have the ability to request from the *Rider* additional medical information as deemed necessary, the expenses of which shall be met by the *Rider*.
- 65.** Until the review process has been completed, the decision of the UCI remains in effect.
- 66.** If *WADA* reverses the decision of the UCI, the reversal shall not apply retroactively. The *Rider* shall not be entitled to compensation by the UCI, in particular for missed participations in *Events*.
- 67.** The decision by *WADA* reversing the decision by the UCI may be appealed exclusively to the CAS by the UCI.
- 68.** If *WADA* confirms the decision by the UCI, the *Rider* may appeal the decisions by UCI and *WADA* exclusively to the CAS.
- 69.** The time limit for appeal with the CAS is 1 (one) month following receipt of the decision by *WADA*.

Review by WADA on its own initiation

- 70.** *WADA* may, on its own initiation, review and reverse at any time the decision by which the TUEC of the UCI denies or withdraws a TUE.
Articles 64 to 69 shall apply.
- 71.** *WADA* may, on its own initiation, review and reverse at any time the grant of a TUE by the UCI.

- 72.** The decision by WADA reversing the grant of the TUE by the UCI may be appealed exclusively to the CAS by the *Rider* or the UCI.
- 73.** The time limit for appeal with the CAS is 1 (one) month following receipt of the decision by WADA.

V

Chapter WHEREABOUTS INFORMATION

Registered Testing Pool

- 74.** The Anti-Doping Commission shall identify a UCI *Out-of-Competition Registered Testing Pool* of those *Riders* who are required to provide up-to-date whereabouts information to the UCI.

Comment: only the Riders included in the Registered Testing Pool are required to provide whereabouts information; however any Rider may be tested Out-of-competition at any time and at any place, even if he is serving a period of Ineligibility.

- 75.** The Anti-Doping Commission shall define the criteria for *Riders* to be included in the *Registered Testing Pool* and may also include *Riders* individually. The Anti-Doping Commission may revise the *Registered Testing Pool* from time to time as appropriate.
- 76.** A *Rider* continues to be included in the *Registered Testing Pool* and to be required to provide up-to-date whereabouts information to the UCI until he has been informed by the Anti-Doping Commission that he has been removed from the *Registered Testing Pool*. *Riders* that serve a period of *Ineligibility* continue to be included in the *Registered Testing Pool* and to be required to provide whereabouts information.
- 77.** A *Rider* who has given notice of retirement from cycling to the UCI may not resume competing at international level unless he notifies the UCI at least 6 (six) months in advance before he expects to return to international competition and is available for unannounced *Out-of-Competition Testing*, at any time during the period before actual return to competition.

Notice of retirement is effective only when the *Rider* has returned his license to his National Federation for that purpose.

Whereabouts information requirements

- 78.** The Anti-Doping Commission shall inform each *Rider* in the *Registered Testing Pool* via written notice that he has been included in the pool and must provide accurate whereabouts information in accordance with these Anti-Doping Rules and with any further instructions that the Anti-Doping Commission may deem appropriate.

UCI CYCLING REGULATIONS

The notice shall set the deadline for when the *Rider* is required to submit whereabouts information. This deadline shall be no earlier than 3 (three) weeks after sending the notice and no later than 2 (two) weeks prior to the start of the quarter.

The *Rider* shall confirm written receipt of such notice directly to the Anti-Doping Commission.

- 79. Each *Rider* in the *Registered Testing Pool* shall file quarterly reports with the Anti-Doping Commission on forms provided by the UCI which specify on a daily basis the locations and times where the *Rider* will be residing, training and competing.
- 80. Should a *Rider's* plans change from those originally submitted on the whereabouts information forms, the *Rider* shall immediately send updates of all information required in the form so that it is current at all times.
- 81. The *Rider* shall send his whereabouts information and updates to the Anti-Doping Commission by fax. The *Rider* may also provide whereabouts information and updates by electronic means, once an appropriate electronic system is in place and the *Rider* is informed accordingly.

Failure to comply

- 82. If whereabouts information is not received in time or is incomplete or inadequate or inaccurate, the Anti-Doping Commission shall provide a written warning to the *Rider*.

Should the *Rider* after the written warning not provide accurate whereabouts information within 7 (seven) days, the *Rider* shall be provided an additional written warning.

- 83. A failure to comply with a written warning shall be recorded, unless the Anti-Doping Commission accepts a reasonable justification.

- 84. Should the *Rider* not be located for *Testing* based on the most up-to-date information received from the *Rider*, the Anti-Doping Commission shall report the missed test, in writing, to the *Rider*.

The *Rider* shall have the right to provide a written explanation and to prove circumstances within 10 (ten) days from the sending of the report.

If the Anti-Doping Commission, after having considered the explanations and evidence provided by the *Rider*, finds the explanation not justified, it shall record a missed test and inform the *Rider*.

- 85. For each attempt to locate the *Rider* for *Testing*, the *Anti-Doping Inspector* shall visit all locations during the times specified by the *Rider* for that date and shall stay 1 (one) hour at each location (or until such moment that the *Rider* had announced to stay at that location, if earlier).

- 86. Should, in a rolling period of 18 (eighteen) months, a *Rider* receive 3 (three) recorded warnings for failure to provide accurate whereabouts information or a combination of failure to provide accurate whereabouts information and missed tests equals 3 (three), an anti-doping violation has occurred.

UCI CYCLING REGULATIONS

The Anti-Doping Commission will notify the *Rider's* National Federation and request it to initiate disciplinary proceedings for an anti-doping violation under article 15.4.

87. *Rider's* failure to provide accurate whereabouts information and/or missed tests under these Anti-Doping Rules may be combined with failures to provide accurate whereabouts information and/or missed tests recorded by other *Anti-Doping Organizations*, provided (i) the *Anti-Doping Organization* had authority under the *Code*, (ii) the Anti-Doping Commission was informed in time and (iii) the facts recorded by the *Anti-Doping Organization* constitute, to the satisfaction of the Anti-Doping Commission, a failure to provide accurate whereabouts information or a missed test under these Anti-Doping Rules.

Coordination with Anti-Doping Organizations

88. The UCI may collect whereabouts information also with the National Federations, WADA and other *Anti-Doping Organizations*.
89. The UCI will make the list of *Riders* in the *Registered Testing Pool* available to WADA. The UCI may make the list available to other *Anti-Doping Organizations*.
90. The UCI will submit all whereabouts information to WADA and WADA will make this information accessible to other *Anti-Doping Organizations* having authority to test the *Rider* as provided in article 8.
91. The UCI may provide whereabouts information to other *Anti-Doping Organizations* having authority to test the *Rider* as provided in article 8.

Confidentiality

92. The UCI shall maintain whereabouts information in strict confidence at all times and shall use it exclusively for purposes of planning, coordinating or conducting *Testing*. The UCI shall destroy the whereabouts information after it is not longer relevant for these purposes.
93. Under the *Code*, WADA and all *Anti-Doping Organizations* having accepted the *Code* are bound to the same obligations concerning confidentiality of whereabouts information.

However, the UCI shall not be liable for the use that WADA or any *Anti-Doping Organization* makes of whereabouts information, even if the information was provided by the UCI. The *Rider* has no claim against the UCI in this respect.

Obligations of National Federations

94. National Federations shall assist the UCI in obtaining whereabouts information concerning *Riders* as requested by the UCI.

- 95.** Each National Federation shall assist its *National Anti-Doping Organization* in establishing the latter's registered testing pool of national level *Riders*.

VI

Chapter TESTING

Procedural Guidelines

- 96.** The Anti-Doping Commission shall issue *Procedural Guidelines* for all aspects of *Testing* conducted under these Anti-Doping Rules.

Procedural Guidelines shall be in conformity with these Anti-Doping Rules and in substantial conformity with the *International Standard for Testing*.

Procedural Guidelines shall be binding upon their approval by the President of the UCI.

- 97.** In addition, the President of the Anti-Doping Commission and the *Anti-Doping Inspector* may at any time impose measures which are urgently required to ensure that *Testing* can take place.

Management

- 98.** Except as provided in articles 107, 108, 109 and 153, *Testing* is conducted by an *Anti-Doping Inspector* and a *Medical Inspector*.

(text modified on 1.01.07).

- 99.** The *Anti-Doping Inspector* is responsible for the on site management of the *Testing*.

- 100.** For World Championships, the Management Committee shall appoint a UCI official doctor who shall be a member of the Anti-Doping Commission or a doctor proposed by the Anti-Doping Commission. The UCI official doctor is responsible for the on site management of the *Testing* at World Championships. He may act as *Medical Inspector*.

- 101.** The *Anti-Doping Inspector* is appointed by the Anti-Doping Commission or, in case of urgency, by its President or his replacement.

However, the *Anti-Doping Inspector* shall be appointed by the National Federation of the country in the following cases:

1. for *Post-Competition Testing* at Events on the B list according to article 112, c;
2. for *Post-Competition Testing* at Events at which the National Federation is authorized to test by the UCI according to article 3;

UCI CYCLING REGULATIONS

3. for *Out-of-Competition Testing* that the National Federation is authorized to conduct on *Riders* by the UCI.
- 102.** The *Medical Inspector* shall be responsible for the sample taking as described in these Anti-Doping Rules and in the *Procedural Guidelines*.
- 103.** The *Medical Inspector* for *In-Competition Testing* shall be a doctor.
- 104.** For *In-Competition Testing*, the *Medical Inspector* shall be appointed by the organizer's National Federation. The race doctor shall not be appointed as *Medical Inspector* for *Testing* at the *Event*.
- 105.** The organizer's National Federation shall also appoint a female nurse to attend the delivery of the sample from women if the *Medical Inspector* is a man and a male nurse to attend the delivery of the sample from men if the *Medical Inspector* is a woman.
- 106.** If needs be and without prejudice to the responsibility of the National Federation, the *Anti-Doping Inspector* may appoint a *Medical Inspector* and/or a nurse on the spot.
- 107.** For *Out-of-Competition Testing*, a *Medical Inspector* is not required to be appointed and the tasks and responsibilities otherwise entrusted to the *Medical Inspector* shall be carried out by the *Anti-doping Inspector*.

(text modified on 1.01.07).

- 108.** The Anti-Doping Commission and, in the case of article 7, the National Federation may arrange to have *Out-of-Competition Testing* carried out by another *Anti-Doping Organization* or by a specialist institute or company. The tasks of the *Anti-Doping Inspector*, *Medical Inspector* and person witnessing the delivery of the sample shall be carried out by the persons or the single person designated for such a purpose by the organization, institute or company in question.

(text modified on 1.01.07).

- 109.** Article 108 shall also apply where a National Federation is authorized to conduct *In-Competition Testing*, unless decided otherwise by the Anti-Doping Commission.
- 110.** The *Medical Inspector* may be assisted by another doctor or a nurse.
- 111.** For the taking of *Samples* other than urine *Samples* any person with the appropriate qualifications may be appointed.

Post-Competition Testing

- 112.** A *Post-Competition Testing* session shall be organized at the following *Events*:
 - a) World Championships, Continental Championships and Regional Games, as per the *Procedural Guidelines*;

- b) World record and continental record attempts;
- c) Any other *Event* designated by the Anti-Doping Commission; these *Events* shall be included on the A list or the B list, depending on whether the *Anti-Doping Inspector* is appointed by the Anti-Doping Commission (A list) or the National Federation of the organizer (B list), in accordance with article 101.

113. At stage races where *Testing* takes place, a *Post-Competition Testing* session shall be organized following each stage unless determined otherwise by the Anti-Doping Commission.

114. At six-day events, *Post-Competition Testing* shall be organized on a minimum of 2 (two) days.

National Federation

115. The National Federation of the organizer of the *Event* shall be responsible for the practical aspects of the organization of the *Post-Competition Testing* session, including the obligations on the organizer. It must insure that all staff and all infrastructure and equipment are available so that *Testing* can be carried out in accordance with these Anti-Doping Rules and the *Procedural Guidelines*.

116. Without prejudice to the application of article 12.1.008 of the Cycling Regulations to the organizer, in the event of negligence in the practical organization of the *Testing* session the National Federation of the organizer shall be liable to a fine not exceeding 10'000 CHF. In *Events* which last more than one day, the fine may be multiplied by the number of days for which the negligence continues.

117. If, as a result of negligence during the practical organization of the *Testing* session, the *Anti-Doping Inspector* appointed by the Anti-Doping Commission is unable to carry out his mission properly, the National Federation and the organizer shall be jointly and severally liable to repay his expenses.

Doping control station

118. Premises suitable for the taking of *Samples* must be provided in the immediate vicinity of the finish line. The location must be clearly signposted from the finish line.

119. Should the circumstances justify it, the Anti-Doping Commission may grant an exemption to the requirement of immediate vicinity. The organizer or his National Federation must submit a fully documented request to the Anti-Doping Commission no later than one month before the start of the *Event*.

120. At the request of the *Anti-Doping Inspector*, the organizer shall appoint an official to protect the entrance of the doping control station and prevent access by persons not involved in *Testing*.

Selection of Riders to be tested

121. The *Riders* to be tested shall be as designated in the *Procedural Guidelines*.

The Anti-Doping Commission may also issue confidential instructions to the *Anti-Doping Inspector* for the selection of *Riders* to be tested in a particular *Event*.

UCI CYCLING REGULATIONS

- 122.** For each *Competition* or *Race* for which a *Post-Competition Testing* session is organized, the *Anti-Doping Inspector* shall draw lots for a first and a second reserve *Rider* who will be subjected to testing in that order if a *Rider* drawn by lot is required to undergo tests as a result of his placing or if a *Rider* meets two criteria for selection simultaneously or if one such *Rider* is unable for practical reasons to undergo *Sample* taking, so that the number of tests called for by the Anti-Doping Commission is carried out.

The reserve *Riders* must check in for *Sample* collection within the prescribed time limit, even if they would not be required to submit to *Sample* collection.

- 123.** The circumstances that another *Rider* was tested than those selected according to the *Procedural Guidelines* or the instructions of the Anti-Doping Commission, shall not invalidate the *Testing* of that *Rider*.

Notification of Riders

- 124.** Any *Rider* including any *Rider* who has abandoned the *Race*, shall be aware that he may have been selected to undergo *Testing* after the *Race* and is responsible for ensuring personally whether he is required to appear for *Sample* collection.

To this end, the *Rider*, immediately after finishing or abandoning the *Race* shall locate and proceed to the place where the list of *Riders* who are required to appear for *Sample* collection, is displayed and consult the list.

Riders who participate in time trials shall consult the list after the last *Rider* finishes his ride.

- 125.** The organizer and the *Anti-Doping Inspector* shall ensure that a list of the *Riders* who are required to appear for *Sample* collection shall be displayed at the finish line and at the entrance of the doping control station before the finish of the winner.

- 126.** At track *Events*, one copy of the list shall be displayed at the entrance of the access tunnel when leaving the track center and the other copy shall be displayed at the entrance of the doping control station.

At trial and indoor *Events*, one copy of the list shall be displayed where the *Riders* leave the course or field after having finished their performance and the other copy shall be displayed at the entrance of the doping control station.

- 127.** *Riders* shall be identified on the list by their name or their race number or their place in the ranking.

- 128.** No *Rider* may take the absence of his name, race number or placing from the displayed list as excuse if he is identified in another manner or if it is established that he had learnt in another way that he was required to appear for *Sample* collection.

- 129.** At all times a *Rider* may be called for *Testing* at a *Post-Competition Testing* session in the same way as for *Individual Testing*.

Comment: In addition to displaying the list of Riders to be selected, additional forms of notification are sometimes used such as audio announcements via "Radio Tour" or at the finish venue. The absence of such additional forms of notification may never be interpreted as an indication that no Testing will take place and is no excuse for failing to submit to Sample taking.

When a Rider does not appear for Sample taking, there is no obligation for the Anti-Doping Inspector, the organizer or anyone else to try to contact or notify the Rider.

- 130.** An escort shall remain close to the Rider and observe him at all times, and accompany him to the doping control station. The absence of an escort cannot be pleaded as a defense.

Time-limit for attendance

- 131.** Each Rider to be tested must present himself at the doping control station within 30 (thirty) minutes of finishing the Race or, where appropriate, within 30 (thirty) minutes of the end of an official ceremony in which he has taken part. For a Rider required to attend a press conference under a provision of the regulations, the deadline shall be extended to 50 (fifty) minutes.

- 132.** A Rider who has abandoned the Race must attend within 30 (thirty) minutes of the finishing time of the last classified Rider.

- 133.** A Rider who has to take part in another Race on the same day may, within the time-limit set as above, ask permission from the Anti-Doping Inspector to submit to Sample taking after the other Race. The inspector shall decide whether the test should take place immediately or following the other Race.

Report

- 134.** For each Post-Competition Testing session, the Anti-Doping Inspector shall draw up a report in which he shall attest that Testing complies with these Anti-Doping Rules and the Procedural Guidelines or note the irregularities that he observed.

Within 48 (forty-eight) hours of the dispatch of the Samples to the laboratory this report must be sent to the Anti-Doping Commission.

Individual Testing

- 135.** Individual Testing may be organized In-Competition and Out-of-Competition, at any time and any place and without notice.

- 136.** The Anti-Doping Commission shall determine the place and time and the Riders to be tested or authorize the National Federation to do so.

UCI CYCLING REGULATIONS

- 137.** *Individual Testing* may be carried out at any place where the privacy of the *Rider* is ensured and that is used solely as a doping control station for the duration of the *Sample* collection session.

Sample taking shall be carried out in the best possible manner under the circumstances given and as discreetly as possible.

Notification of Riders

- 138.** *No Advance Notice Testing* shall be the notification method for *Individual Testing* wherever possible.

- 139.** *Riders* shall be called for *Individual Testing* using a notification form.

- 140.** The *Anti-Doping Inspector* shall notify the *Rider* in person. When the notification takes place *In-Competition*, the *Rider* may also be notified via his team manager or club representative.

- 141.** The *Rider* or his team manager or club representative shall sign the original notification form as proof of receipt. If the *Rider* or his team manager or club representative refuses to sign that he has been notified or evades notification, the *Anti-Doping Inspector* shall note this on the form and inform the *Rider* of the consequences of failing to comply if possible.

- 142.** During stage races and World Championships, the team manager or club representative must always be in a position to indicate where his *Riders* are in order that they may be contacted as quickly as possible.

Team managers or club representatives who give incorrect information, refuse to give information or obstruct *Testing* in any other way shall commit an anti-doping violation under article 15.5 (*Tampering or Attempting to tamper*).

- 143.** The *Rider* notified of *No Advance Notice Testing* shall remain within sight of the *Anti-Doping Inspector* or a chaperone at all times from the moment of in-person notification until the completion of the *Sample* collection procedure. If it was not possible for the *Anti-Doping Inspector* to observe the *Rider* or to have the *Rider* chaperoned at all times, this shall be recorded by the *Anti-Doping Inspector*.

- 144.** The time-limit within which the *Rider* is to appear for *Sample* taking shall be set by the *Anti-Doping Inspector*, taking account of the circumstances. *Sample* taking shall be carried out as soon as possible and, except in abnormal circumstances, not later than one hour of the *Rider* (or his team manager or club representative) receiving notification. The *Anti-Doping Inspector* may accept a request from a *Rider* to perform urgent preferential activities before proceeding to the doping control station. The request shall be rejected if it will not be possible for the *Rider* to be continuously observed.

Common rules for Post-Competition Testing and Individual Testing

Attendants

- 145.** The *Rider* may be accompanied by a person of his choice and an interpreter during the *Sample* collection session except when the *Rider* is passing an urine *Sample*.

- 146.** A *Minor Rider*, and the witnessing doctor or nurse are entitled to have a representative observe the doctor or nurse when the *Minor Rider* is passing a urine *Sample*, but without the representative directly observing the passing of the *Sample* unless requested to do so by the *Minor Rider*.
- 147.** When applicable under the *Independent Observer Program*, WADA Independent Observers may attend the *Sample* taking session. WADA Independent Observers shall not directly observe the passing of the *Sample*.
- 148.** The *Rider*, his attendant and the interpreter and any objects they bring with them may be searched.

Time limit for *Sample* taking

- 149.** When a *Rider* does not report to the doping control station within the time-limit, the *Anti-Doping Inspector* shall use his judgment whether to attempt to contact the *Rider*.
- 150.** If a *Rider* foresees that he might be prevented from reporting within the time-limit, he shall try, by all available means, to inform the *Anti-Doping Inspector*.
- 151.** At a minimum, the *Anti-Doping Inspector* and the *Medical Inspector* shall wait 30 (thirty) minutes after the time-limit before departing.
- 152.** If the *Rider* reports to the doping control station after the minimum waiting time and prior to the departure of the *Anti-Doping Inspector* and the *Medical Inspector*, they shall if at all possible proceed with collecting a *Sample* and shall document the details of the delay in the *Rider* reporting to the doping control station.
- 153.** If only one of the *Anti-Doping Inspector* or *Medical Inspector* is still present, *Sample* collection performed by only one of them may take place if both the *Rider* and the *Anti-Doping Inspector* or *Medical Inspector* so agree.
- 154.** The *Sample* taking may not be delayed, for example to await the arrival of the *Rider's* attendant or interpreter.
- 155.** The *Rider* shall only leave the doping control station if authorized by the *Anti-Doping Inspector* and under continuous observation by the *Anti-Doping Inspector* or a chaperone. The *Anti-Doping Inspector* shall consider any reasonable request by the *Rider* to leave the doping control station, until the *Rider* is able to provide a *Sample*.

If the *Anti-Doping Inspector* gives approval for the *Rider* to leave the doping control station, the *Anti-Doping Inspector* shall agree with the *Rider* on:

- a) The purpose of the *Rider* leaving the doping control station; and
- b) The time of return (or return upon completion of an agreed activity).

The *Anti-Doping Inspector* shall document this information and the actual time of the *Rider's* departure and return.

UCI CYCLING REGULATIONS

- 156.** The *Anti-Doping Inspector* and *Medical Inspector* shall continue the doping control session until the *Rider* delivers the *Samples* required under these Anti-Doping Rules.
- 157.** Should the *Rider* leave the doping control station, before the *Sample* is taken, he shall be considered to have refused the test and shall incur the sanctions set out in article 15.3.
- 158.** Should a *Rider* leave the doping control station after the *Samples* are taken but before all the formalities are completed, the *Testing* shall be deemed valid.
- 159.** Should the *Anti-Doping Inspector* or the *Medical Inspector* discharge a *Rider* or terminate the *Testing* session before the *Rider* has been tested, the *Rider* concerned shall be considered as not to have been selected for *Sample* taking and shall not have committed an anti-doping violation for having left the doping control station.
- 160.** The events covered by articles 149 to 159 shall be recorded.

Anomalies

- 161.** Any behavior by the *Rider* and/or persons associated with the *Rider* or anomalies with potential to compromise the *Sample* collection shall be recorded.
- 162.** If there are doubts as to the origin or authenticity of the *Sample*, the *Rider* shall be asked to provide an additional *Sample*. If the *Rider* refuses to provide an additional *Sample*, this shall be recorded by the *Anti-Doping Inspector*. A refusal to provide an additional *Sample* shall be considered as a refusal to submit to *Sample* collection under article 15.3.

Documentation

- 163.** The *Anti-Doping Inspector* shall provide the *Rider* with the opportunity to document any concerns he may have about how the session was conducted.
- 164.** The *Rider* and the *Anti-Doping Inspector* shall sign appropriate documentation to indicate their satisfaction that the documentation accurately reflects the details of the *Rider's Sample* collection session, including any concerns recorded by the *Rider*. The *Rider* representative shall sign on behalf of the *Rider* if the *Rider* is a *Minor*. Other persons present who had a formal role during the *Rider's Sample* collection session may sign the documentation as a witness of the proceedings.

The *Anti-Doping Inspector* or the *Medical Inspector* shall provide the *Rider* with a copy of the records of the *Sample* collection session that have been signed by the *Rider*.

- 165.** By appending his signature on the *Testing* form, the *Rider* confirms that, subject to any concern recorded by the *Rider*:
 - 1. the *Testing* was conducted in accordance with applicable standards and regulations;
 - 2. any subsequent complaint is excluded;
 - 3. he received a copy of the *Testing* form.

Reporting

- 166.** The UCI will report all *Testing* conducted under these Anti-Doping Rules to WADA, including the name of the *Rider*, the date and place of the test and whether the test was *In-Competition* or *Out-of-Competition*.

National Federations that conduct *Testing* under these Anti-Doping Rules shall report all *Testing* to the UCI immediately after *Testing*.

WADA shall make the information accessible to the *Rider*, the *Rider's* National Federation, National Olympic Committee or National Paralympic Committee, *National Anti-Doping Organization* and the International Olympic Committee or International Paralympic Committee.

Property of Samples

- 167.** The *Samples* collected under these Anti-Doping Rules shall become the property of the UCI upon collection.

Samples for screening

- 168.** *Riders* shall be also subject to *Sample* taking for screening purposes.
- 169.** The UCI may use for screening purposes any relevant information collected, received or discovered, including blood *Samples* or other non-urine *Samples* collected pursuant to other regulations. The UCI is not obliged to justify the reason why the *Rider* was targeted and what information was used for screening or targeting.

Sample collection equipment

- 170.** *Sample* collection equipment systems shall be used that:
1. have a unique numbering system incorporated into all bottles, containers, tubes or any other item used to seal the *Rider's Sample*;
 2. have a sealing system that is tamper evident;
 3. ensure the identity of the *Rider* is not evident from the equipment itself;
 4. ensure that all equipment is clean and sealed prior to use by the *Rider*.

Sample transport

- 171.** The *Anti-Doping Inspector* shall be responsible for:
- storing the *Samples* prior to transport;
 - sending the *Samples* with accompanying documentation to the laboratory;
 - sending the *Sample* collection session documentation for the laboratory and the Anti-Doping Commission in accordance with the *Procedural Guidelines*.

Costs of Testing

- 172.** The costs of *In-Competition Testing* initiated and directed by the UCI shall be borne by the organizer of the *Event*.

UCI CYCLING REGULATIONS

- 173.** The costs of *Out-of-Competition Testing* directed by the UCI shall be borne by the UCI. The costs of *Out-of-Competition Testing* directed by a National Federation so authorized at its request, shall be borne by that National Federation.
- 174.** The National Federation of the *Rider* shall be responsible for the costs of the B *Sample* analysis.
- 175.** Should a *Rider* be sanctioned following *Testing*, he shall bear the costs incurred for *Out-of-Competition Testing* and B *Sample* analysis.

Analysis of Samples

- 176.** *Samples* shall be sent for analysis only to WADA-accredited laboratories or as otherwise approved by WADA. The choice of the WADA-accredited laboratory (or other method approved by WADA) used for the *Sample* analysis shall be determined exclusively by the Anti-Doping Commission.
- 177.** When specific circumstances so justify, the Anti-Doping Commission may request that part of a *Sample* is analyzed in a second laboratory.
- 178.** *Doping control Samples* shall be analyzed to detect *Prohibited Substances* and *Prohibited Methods* identified on the *Prohibited List* and other substances as may be directed by WADA pursuant to the Monitoring Program described in Article 4.5 of the *Code*.
- 179.** Laboratories shall analyze doping control *Samples* and report results in conformity with the WADA *International Standard for Laboratory Analysis*.
- 180.** Subject to articles 168 and 169, no *Sample* may be used for any purpose other than the detection of substances (or classes of substances) or methods on the *Prohibited List*, or as otherwise identified by WADA pursuant to its Monitoring Program, without the *Rider's* written consent.
- 181.** The laboratory shall report any *Adverse Analytical Findings* to the Anti-Doping Commission and WADA or, if the *Adverse Analytical Finding* concerns World Championships, to the UCI official doctor and WADA.

VII

Chapter RESULTS MANAGEMENT

- 182.** Results management under these Anti-Doping Rules, including results management from a test by a National Federation pursuant to articles 3 and 7, shall be conducted by the UCI's Anti-Doping Commission.
- 183.** The Anti-Doping Commission shall refer results management concerning a *License-Holder* who usually does not participate in *International Events*, to the *License-Holder's* National Federation, who shall conduct results management in substantial conformity with this chapter.

Review

- 184.** If after receiving a test analysis report, an inspector's statement, a referral by another *Anti-Doping Organization*, or any document or information regarding a possible anti-doping violation, the Anti-Doping Commission considers that no anti-doping violation or any other breach of these Anti-Doping Rules has taken place, then the case shall be taken no further.

This decision shall not be definitive and the Anti-Doping Commission may reopen the case on its own initiation.

WADA shall be informed of a decision not to proceed with a case. If WADA so requests, the Anti-Doping Commission shall reopen the case and request the National Federation to instigate disciplinary proceedings in accordance with article 224.

- 185.** Before taking a decision, the Anti-Doping Commission may order additional investigations. National Federations shall be obliged to conduct such investigations as the Anti-Doping Commission may deem appropriate. All *License-Holders* are obliged to assist.

- 186.** Upon receipt of an *A Sample Adverse Analytical Finding*, the Anti-Doping Commission shall conduct a review to determine whether: (a) an applicable Therapeutic Use Exemption has been granted, or (b) there is any apparent departure from these Anti-Doping Rules, the *Procedural Guidelines* or the *International Standards for Testing* or laboratory analysis that undermines the validity of the *Adverse Analytical Finding*.

- 187.** If the initial review under article 186 does not reveal an applicable Therapeutic Use Exemption or departure from these Anti-Doping Rules, the *Procedural Guidelines*, the *International Standard for Testing* or the *International Standard* for laboratory analysis in force at the time of *Testing* or analysis that undermines the validity of the *Adverse Analytical Finding*, the Anti-Doping Commission shall promptly notify the *Rider's* National Federation of: (a) the *Adverse Analytical Finding*; (b) the anti-doping rule violated, or a description of the additional investigation that will be conducted as to whether there is an anti-doping rule violation; (c) the *Rider's* right to promptly request the analysis of the *B Sample* or, failing such request, that the *B Sample* analysis shall be deemed waived; (d) the right of the *Rider* and/or the *Rider's* representative to attend the *B Sample* opening and analysis if such analysis is requested; and (e) the *Rider's* right to request copies of the *A* and *B Sample* laboratory documentation package which includes information as required by the *International Standard* for laboratory analysis.

A copy of the notification may be sent to the *Rider* and/or the *Rider's* club or team.

A copy of the notification is sent to WADA and to the *Rider's* National Anti-Doping Organization.

- 188.** The *Rider's* National Federation shall within 2 (two) working days send a copy of the notification from the Anti-Doping Commission to the *Rider* concerned. It shall also notify the UCI that this copy has been sent.

UCI CYCLING REGULATIONS

- 189.** The notification of the *Rider's* National Federation under article 187 and the notification of the *Rider* under article 188 shall be confirmed by the dispatch of a registered letter with proof of delivery.
- 190.** Any communication by the National Federation will be deemed valid if sent to the most recent address for the *Rider* as notified to the National Federation.

Analysis of B Sample

- 191.** The *Rider* and/or his National Federation and the Anti-Doping Commission shall be entitled to demand the analysis of the B Sample.
- 192.** The request for the analysis of the B Sample shall indicate whether the *Rider* wants not only the opening, but also the analysis of the B Sample to be attended by him or a representative.
- 193.** The request for the analysis of the B Sample shall be made directly to the laboratory by the *Rider's* National Federation, either on its own initiative or at the request of the *Rider*. A copy of the request for the analysis must be sent to the UCI at the same time.
- 194.** To be acceptable, the request for the analysis of the B Sample by the National Federation shall be sent to the laboratory no more than 5 (five) working days after receipt of the registered letter sent to the National Federation of the *Rider* informing it of the A Sample Adverse Analytical Finding.
- 195.** The analysis of the B Sample shall be conducted by the laboratory that conducted the analysis of the A Sample.
- However, if justified by the need to have the B Sample analyzed, the UCI Anti-Doping Commission may decide that the analysis of the B Sample shall be carried out by another laboratory which it shall designate.
- 196.** The analysis of the B Sample may be carried out by 2 (two) laboratories in accordance with article 177. Should the analysis of the A Sample carried out in this fashion have shown that the Adverse Analytical Finding is verifiable in the second laboratory only, the analysis of the B Sample shall be valid if carried out in this laboratory.
- 197.** The opening of the B Sample may be attended by the *Rider*, an expert designated by him or by his National Federation, a representative of the *Rider's* National Federation and a representative of the UCI.
- 198.** The analysis of the B Sample may be attended by the *Rider* or one representative if such request was made when the B Sample analysis was requested. The laboratory may restrict the attendance in order to avoid any disturbance of the analysis.
- 199.** The laboratory, in consultation with the parties involved, sets a date for the analysis of the B Sample in a period of 10 (ten) working days after reception of the request excepted the opposed agreement of the Anti-Doping Commission.

- 200.** The B *Sample* analysis of a blood *Sample* shall take place no later than 3 (three) days after the analysis of the A *Sample*. The Anti-Doping Commission shall inform the *Rider* and/or his National Federation as soon as possible of any A *Sample Adverse Analytical Finding* and of the date set for the B *Sample* analysis by fax or by email. The terms and procedures set above shall not apply for notifying the *Rider*.

The B *Sample* analysis shall be valid, even if the *Rider* did not receive notification in time or was unable to attend or to be represented.

- 201.** No party may claim their inability to attend for the analysis of the B *Sample* on the date set as a reason for invalidating the analysis of the B *Sample*.

- 202.** The *Rider's* National Federation shall be responsible for the costs of the analysis of the B *Sample*.

The *Rider's* National Federation may submit the request for the analysis following payment by the *Rider* of a sum not exceeding CHF 700.

- 203.** A *Rider* may accept the A *Sample* analytical results and waive the B *Sample* analysis. The UCI may nonetheless elect to proceed with the B *Sample* analysis.

- 204.** If the B *Sample* proves negative, the entire test shall be considered negative and the *Rider*, his National Federation, his National Anti-Doping Organization and WADA shall be so informed.

- 205.** If a *Prohibited Substance* or the *Use* of a *Prohibited Method* is identified, the findings shall be reported to the *Rider*, his National Federation, his National Anti-Doping Organization and to WADA.

A copy of the report may be sent to the *Rider's* team or club.

Follow-up investigations

- 206.** The Anti-Doping Commission shall conduct any follow-up investigation as may be required by the *Prohibited List*. Upon completion of such follow-up investigation, the Anti-Doping Commission shall promptly notify the *Rider* regarding the results of the follow-up investigation and whether or not the Anti-Doping Commission asserts that an anti-doping rule was violated.

- 207.** The costs of follow-up investigations are at the expense of the *Rider*. The *Rider* may be requested to advance costs. If the *Rider* refuses to do so, he is presumed to have accepted the analytical results and the case shall proceed accordingly.

Test results management at World Championships

- 208.** During World Championships, the laboratory shall report any *Adverse Analytical Finding* to the UCI official doctor.

If it is not possible for the test results to reach the UCI official doctor before the end of the Championships, they shall be sent to the UCI Anti-Doping Commission.

- 209.** Upon receipt of an *A Sample Adverse Analytical Finding* from a test conducted at the World Championships, the UCI official doctor shall conduct the review described in article 186 and, where applicable, notify the *Rider* or, should this be impossible, his team manager immediately.
- 210.** The UCI official doctor shall without further formalities call for the analysis of the *B Sample*, at which he has the right to be present. He shall inform the *Rider* and the delegation from his National Federation of the place, date and time of the analysis. No postponement of the analysis may be granted.
- 211.** If the *B Sample* analytical result confirms the *A Sample* analytical result, the UCI official doctor shall inform the *Rider*, the president of the commissaires panel, the Anti-Doping Commission and the *Rider's* National Federation.
The notification to the *Rider's* National Federation may be made to the delegation from that Federation attending the Championships.

Results management at stage races and six-days races

- 212.** During a stage race or a six-day race, upon receipt of an *A Sample Adverse Analytical Finding* from a test conducted at that stage race or a six-days race and completion of the review described in article 186, the Anti-Doping Commission shall notify the *Rider* via the president of the commissaires panel.

The president of the commissaires panel shall hear the *Rider's* explanations.

- 213.** The request for the analysis of the *B Sample* shall be submitted in writing to the *Anti-Doping Inspector* within 3 (three) hours of the notification specified in article 212.

The *Rider* shall be issued with a receipt stating the time that the request was submitted.

- 214.** No postponement of the analysis of the *B Sample* may be granted in order to permit attendance by the persons noted in articles 145 and 146 beyond 4 (four) days from the date of the request for the analysis.

- 215.** The *Anti-Doping Inspector* shall submit the *B Sample* analysis report to the president of the commissaires panel.

- 216.** If the *B Sample* analytical result confirms the *A Sample* analytical result, the Anti-Doping Commission shall inform the president of the commissaires panel for the purposes of article 219.

VIII

Chapter PROVISIONAL MEASURES

- 217.** 1. If after the review described in articles 184 to 206 the Anti-Doping Commission asserts that an anti-doping violation under article 15.1 or article 15.2 was committed, the Anti-Doping Commission may ban the *Rider* from participating in *Events* for such time that the violation, in the opinion of the Anti-Doping Commission, is likely to affect the *Rider's* results.
2. Before the ban goes into effect or on a timely basis thereafter, the *Rider* shall be given an opportunity to be heard in either written or oral form. The President of the Anti-Doping Commission may appoint a member of the Anti-Doping Commission, including himself, or an *Anti-Doping Inspector* to hear the *Rider*.
3. The decision shall be taken by the member of the Anti-Doping Commission who has heard the *Rider* or, if the *Rider* was heard by an *Anti-Doping Inspector*, by the President of the Anti-Doping Commission having received the account of the *Anti-Doping Inspector*.

- 218.** If after the initial review described in article 186 and at the eve or during a particular *Event*, the Anti-Doping Commission makes an assertion that an anti-doping violation may have been committed under articles 15.1 or 15.2 and determines that the asserted anti-doping violation, that occurred prior to that *Event*, may affect the *Rider's* results at the *Event*, the Anti-Doping Commission may request that the *Rider* is banned from the *Event*.

The request shall be made to the president of the commissaires panel. The president of the commissaires panel shall summon the *Rider* to be heard and decide whether the *Rider* shall be banned from the *Event*.

- 219.** If, during an *Event* and where applicable after the review described in article 186 the Anti-Doping Commission or, at World Championships, the official doctor asserts that an anti-doping violation has been committed during the same *Event*, the Anti-Doping Commission or the official doctor will inform the president of the commissaires panel for the *Event*. The president of the commissaires panel shall summon the *License-Holder* concerned to be heard. The president of the commissaires panel may *Disqualify* the *Rider* or ban the *License-Holder* from the *Event* subject to the due opinion of the President or another member of the Anti-Doping Commission.

If a *Rider* is *Disqualified* for an asserted violation under articles 15.1 or 15.2 that occurred during a team race, the *Rider's* team shall be relegated to the last place of the race. In stage races on the road and subject to the application of article 279 after the decision of the hearing body, the team will be given its actual time.

- 220.** In case of a recorded warning or a missed test in a period of 45 (forty-five) days before the start of a Major Tour, the *Rider* is not allowed to participate in that Tour.

- 221.** The provisional measures under articles 217, 218 and 219 may be combined.

Such provisional measures may also be imposed on *Rider's Support Personnel* against whom an anti-doping violation is asserted for any period prior to the decision of the hearing body.

- 222.** The provisional measures stipulated above are intended to preserve fair competition. They shall not prejudice the decision whether an anti-doping violation has occurred and shall not give rise to any claim in the event that the *License-Holder* is acquitted.
- 223.** Provisional measures regarding a *License-Holder* who has been referred to his National Federation according to article 183, shall be governed by the rules of the National Federation. Under all circumstances such *License-Holder* shall be *Disqualified* from participation in an *International Event* prior to the decision of the hearing body.

IX

Chapter RIGHT TO A FAIR HEARING

- 224.** When, following the results management process described in chapter VII, the Anti-Doping Commission makes an assertion that these Anti-Doping Rules have been violated, it shall notify the *License-Holder's* National Federation and request it to instigate disciplinary proceedings. It shall also send a copy of the test analysis report and/or other documentation. A copy of the statement may be sent to the *License-Holder* and/or the *License-Holder's* club or team.

A copy of the statement is sent to WADA and to the *License-Holder's* National Anti-Doping Organization.

***License-Holder* called before his National Federation**

- 225.** The *License-Holder's* National Federation shall call the *License-Holder* before it to hear his grounds and explanations.

This summons must be sent within 2 (two) working days of the receipt of the statement under Article 224.

- 226.** The summons shall be sent by registered letter. It shall indicate the nature of the case against the *License-Holder*. The summons must be accompanied by a copy of the test analysis reports and documents received by the Federation from the Anti-Doping Commission. If these enclosures are missing, the *License-Holder* must notify the National Federation without delay.

- 227.** The summons must be sent at least 10 (ten) days before the hearing to which the *License-Holder* has been called. A copy of the summons shall be sent to the UCI at the same time.

The summons shall indicate the date, time and venue for the hearing.

228. A single postponement to the hearing may be granted of not more than 8 (eight) days, except where the party concerned establishes a case of force majeure.

229. The *License-Holder* may forgo the hearing in which event the case will be conducted in writing.

Rights of the defense

230. The *License-Holder* shall be heard and the case investigated by the competent hearing body of the *License-Holder's* National Federation in accordance with the regulations of the *License-Holder's* National Federation whilst taking account of the following articles:

231. The hearing body shall be fair and impartial.

232. At the request of one of the parties to the case or at their own request the following shall also be heard: the organiser's National Federation, the laboratory which carried out the analysis, the *Anti-Doping Inspector*, the *Medical Inspector*, witnesses and expert witnesses.

The interested party shall be responsible for calling these persons to the proceedings and for their costs. It shall at the same time notify the other parties and the competent body.

233. The UCI may give its opinion in each case and demand that a sanction be imposed, either in writing or at the hearing.

It may call for a copy of the documentation of the case in full, including the proceedings of the hearing and the documents submitted by the parties.

234. The parties must provide each other with all the statements and documents which they intend to submit at the earliest opportunity. They shall also send them to the UCI at the same time.

235. The *License-Holder* has the right to see the contents of the case files. Each party may obtain a copy at its own expense.

Furthermore the case files may be consulted during the hearing.

236. The hearing shall be public unless the *License-Holder* requests otherwise.

The president of the hearing body may also as of right prohibit public access to the room during all or part of the hearing in the interest of public order or when the protection of privacy or medical secrecy justifies it.

237. Each party shall have the right to be represented by a qualified lawyer or by a representative on presentation of authorization in writing. The parties may be aided by any other person of their choice.

- 238.** Each party and any witnesses and expert witnesses called shall be heard subject to the hearing body's discretion to accept testimony by telephone or written submission. The *License-Holder* shall have the right to speak last.
- 239.** In the event that a party who has been convened should fail to appear, the case will be heard in that party's absence. The decision shall be deemed to have been taken after due hearing of the parties.
- 240.** If the hearing body determines that the *License-Holder* has insufficient knowledge of the language of the proceedings, the *License-Holder* has the right to an interpreter at the hearing. The hearing body will determine the identity and responsibility for the cost of the interpreter.
- 241.** Each party is responsible for the costs of interpreters for its witnesses and experts.

Decision

- 242.** The decision of the hearing body shall note the identities of the parties called or heard and shall contain a brief summary of the procedure.

It shall bear the names of the persons who took the decision and must be signed by them.

- 243.** The decision shall be dated and reasoned.

It shall, where appropriate, indicate the *Prohibited Substances* or *Prohibited Methods* for which the *Rider* has been declared positive.

It shall indicate the sanctions imposed on the *License-Holder*.

Costs

- 244.** Subject to article 245 and in the absence of a specifically justified decision, each party shall bear the costs which it incurs.

- 245.** If the *License-Holder* is found guilty of an anti-doping violation, he shall bear:
1. The cost of the proceedings as determined by the hearing body.
 2. The cost of the result management by the Anti-Doping Commission; the amount of this cost shall be CHF 1000, unless a higher amount is claimed by the UCI and determined by the hearing body.
 3. The cost of the B Sample analysis, where applicable. The National Federation shall be jointly and severally liable for its payment to the UCI.
- The *License-Holder* shall owe the costs under 2) and 3) also if they were not awarded in the decision.

- 246.** Should the *License-Holder* be acquitted the party nominated in the decision shall be liable for the costs under article 245.2.

Notification of the decision

- 247.** One full copy of the decision, signed at least by the president of the hearing body, shall be sent to the *License-Holder* and the UCI. These copies shall be sent by registered post with proof of receipt within 3 (three) working days of the decision. The UCI shall send the text of the decision to WADA and to the *License-Holder's National Anti-Doping Organization*.

Exclusion of an appeal at national level

- 248.** The decision by the hearing body of a *License-Holder's National Federation* shall not be subject to an appeal before another body (appeals board, tribunal, etc.) at National Federation level.

If such an appeal is entered, it must be declared inadmissible. Any other decision is void as of right. However, the UCI may ask the Court of Arbitration for Sport (CAS) to pronounce nullity where appropriate upon supplementary application in an appeal procedure against the decision of the competent body. This application may be made at any time during the procedure before the CAS.

Duration of the proceedings

- 249.** The National Federation shall keep the UCI fully informed as to the status of the case.
- 250.** The proceedings before the hearing body of the *License-Holder's National Federation* must be completed within 1 (one) month from the time limit set for the dispatch of the summons.

The National Federation shall be penalized by the disciplinary commission, incurring a fine of CHF 5000 for each week's delay without prejudice to the obligation to complete proceedings as fast as possible.

- 251.** If the completion of the hearing is delayed beyond three months, the UCI may elect to bring the case directly to a single arbitrator from the Court of Arbitration for Sport (CAS), acting as a first instance tribunal. The case shall be handled in accordance with the Court of Arbitration for Sport appeal procedure without reference to any time limit for appeal. The *License-Holder's National Federation* shall be summoned to participate in the proceedings and shall bear all costs resulting for all parties from bringing the proceedings from the National Federation to the CAS.

- 252.** The *License-Holder* may forego a hearing by acknowledging the violation of these Anti-Doping Rules and accepting *Disqualification*, *Ineligibility* and costs consistent with these Anti-Doping Rules as proposed or accepted by the Anti-Doping Commission.

- 253.** If any new fact is revealed of a nature which might alter the decision issued by the hearing body of the *License-Holder's National Federation* after the date of pronouncement, the interested party may request that the case be reopened before the National Federation, unless it is possible to raise the new issue in existing proceedings before the CAS.

The new evidence must predate the decision of the hearing body, and the party submitting it must establish that it could not have known about it prior to the hearing where the decision was issued.

The request to reopen the case must be made within one month of the party's becoming aware of the evidence in question or it shall be debarred. The burden of proof regarding this date shall lie with the party submitting the new evidence.

- 254.** Articles 248, 250 and 251 shall not apply to the *License-Holder* that was referred to his National Federation according to article 183.

X

Chapter SANCTIONS AND CONSEQUENCES

- 255.** These Anti-Doping Rules concerning sanctions and consequences shall be construed and implemented in compliance with human rights and general principles of law, among which proportionality and individual case management.

Automatic Disqualification of individual results

- 256.** A violation of these Anti-Doping Rules in connection with an *In-Competition* test automatically leads to *Disqualification* of the individual result obtained in that *Competition*.

Disqualification of Results in Event during which an Anti-Doping Rule Violation occurs

- 257.** Except as provided in articles 258 and 259, an anti-doping rule violation occurring during or in connection with an *Event* leads to *Disqualification* of the *Rider's* individual results obtained in that *Event* according to the following rules:

1. If the violation is a violation of
 - a) article 15.5 (*Tampering or Attempting to tamper*), or
 - b) article 15.6 (*Possession*), or
 - c) article 15.7 (*Trafficking*), or
 - d) article 15.8 (*Administration, Attempted administration or any type of complicity*),all of the *Rider's* individual results are disqualified.
2. If the violation involves
 - a) the presence, *Use* or *Attempted Use* of a *Prohibited Substance* or a *Prohibited Method* (articles 15.1 and 15.2), other than a *Specified Substance*; or
 - b) evading *Sample* collection or refusing to submit to *Sample* collection (article 15.3); or
 - c) failing to submit to *Sample* collection (article 15.3), except when the *Rider* establishes that he bears *No Significant Fault or Negligence*;

all of the *Rider's* results are disqualified, except for the results obtained (i) in *Competitions* prior to the *Competition* in connection with which the violation occurred and for which the *Rider* was tested with a negative result, and (ii) in *Competitions* prior to the *Competition(s)* under point 1.

3. If the violation involves the presence, *Use* or *Attempted Use* of a *Specified Substance*, all of the *Rider's* results obtained in *Competitions* posterior to the *Competition* in connection with which the violation occurred are disqualified, except for those results which were not likely to have been affected by the violation.
 4. If the violation is a failure to submit to *Sample* collection and if the *Rider* establishes that he bears *No Significant Fault or Negligence*, the *Rider's* results obtained in other *Competitions* shall not be disqualified.
- 258.** If the anti-doping violation involves the presence, *Use* or *Attempted Use* of a *Prohibited Substance* or a *Prohibited Method* (article 15.1 and 15.2) and the *Rider* establishes that he bears *No Fault or Negligence*, his individual results in the other *Competitions* shall not be disqualified except to the extent that they were likely to have been affected by the *Rider's* anti-doping violation.
- 259.** 1. If the *Event* is a stage race, an anti-doping violation committed in connection with any stage, entails *Disqualification* from the *Event*, except when (i) the anti-doping violation involves the presence, *Use* or *Attempted Use* of a *Prohibited Substance* or a *Prohibited Method*, (ii) the *Rider* establishes that he bears *No Fault or Negligence* and (iii) his results in no other stage were likely to have been influenced by the *Rider's* anti-doping violation.
2. If the anti-doping violation committed in a stage race involves the presence, *Use* or *Attempted Use* of a *Specified Substance* and only a warning and reprimand are imposed, *Disqualification* from the *Event* is optional.
- If the *Rider* is not *Disqualified* from the *Event*, 1% (one percent) of the time recorded by the *Rider* during the stage on which he tested positive shall be added to the final time on the individual classification. The number of points scored during that same stage shall be deducted from the final classification. Any prize won in connection with the stage in which the anti-doping violation occurred shall be forfeited.
- 260.** In those cases that are not considered under articles 257 to 259, the *Disqualification* of the *Rider's* individual results obtained on the *Event* is optional.

Imposition of Ineligibility for Prohibited Substances and Prohibited Methods

- 261.** Except for the specified substances identified in article 262, the period of *Ineligibility* imposed for a violation of article 15.1 (presence of *Prohibited Substance* or its *Metabolites* or *Markers*), article 15.2 (*Use* or *Attempted Use* of *Prohibited Substance* or *Prohibited Method*) and article 15.6 (*Possession of Prohibited Substances and Methods*) shall be:

First violation: 2 (two) years' *Ineligibility*

Second violation: Lifetime *Ineligibility*

However, the *License-Holder* shall have the opportunity in each case, before a period of *Ineligibility* is imposed, to establish the basis for eliminating or reducing this sanction as provided in articles 264 and 265.

Specified Substances

262. Where a *Rider* can establish that the Use of a *Specified Substance* was not intended to enhance sport performance, the period of *Ineligibility* found in article 261 shall be replaced with the following:

First violation: At a minimum, a warning and reprimand and no period of *Ineligibility* from future *Events*, and at a maximum, 1 (one) year's *Ineligibility*.

Second violation: 2 (two) years' *Ineligibility*

Third violation: Lifetime *Ineligibility*

However, the *License-Holder* shall have the opportunity in each case, before a period of *Ineligibility* is imposed, to establish the basis for eliminating or reducing (in the case of a second or third violation) this sanction as provided in articles 264 and 265.

Ineligibility for other Anti-Doping Rule Violations

263. The period of *Ineligibility* for other violations of these Anti-Doping Rules shall be:

1. For violations of article 15.3 (evading *Sample* collection, refusing or failing to submit to *Sample* collection) or article 15.5 (*Tampering with Doping Control or Attempting*), the *Ineligibility* periods set forth in article 261 shall apply.
2. For violations of article 15.7 (*Trafficking*) or article 15.8 (administration of *Prohibited Substance* or *Prohibited Method*), the period of *Ineligibility* imposed shall be a minimum of 4 (four) years up to lifetime *Ineligibility*. An anti-doping rule violation involving a *Minor* shall be considered a particularly serious violation, and, if committed by *Rider Support Personnel* for violations other than specified substances referenced in article 262, shall result in lifetime *Ineligibility* for such *Rider Support Personnel*. In addition, violations of such articles which also violate non-sporting laws and regulations, may be reported to the competent administrative, professional or judicial authorities.
3. For violations of article 15.4, as defined in article 86, (*Whereabouts Violations or Missed Tests*), the period of *Ineligibility* shall be:

First violation: 3 (three) months to 1 (one) year *Ineligibility*.

Second and subsequent violations: 1 (one) year to 2 (two) years' *Ineligibility*.

Elimination or Reduction of Period of *Ineligibility*

- 264.** If the *Rider* establishes in an individual case involving an anti-doping rule violation under article 15.1 (presence of *Prohibited Substance* or its *Metabolites* or *Markers*) or *Use of a Prohibited Substance* or *Prohibited Method* under article 15.2) or an anti-doping violation under article 15.6 (*Possession of Prohibited Substances* or *Methods*) that he bears *No Fault or Negligence* for the violation, the otherwise applicable period of *Ineligibility* shall be eliminated. When a *Prohibited Substance* or its *Markers* or *Metabolites* is detected in a *Rider's Specimen* in violation of article 15.1 (presence of a *Prohibited Substance*), the *Rider* must also establish how the *Prohibited Substance* entered his system in order to have the period of *Ineligibility* eliminated. In the event this article is applied and the period of *Ineligibility* otherwise applicable is eliminated, the anti-doping rule violation shall not be considered a violation for the limited purpose of determining the period of *Ineligibility* for multiple violations under articles 261, 262 and 269-271.
- 265.** This article 265 applies to anti-doping rule violations involving article 15.1 (presence of a *Prohibited Substance* or its *Metabolites* or *Markers*), *Use of a Prohibited Substance* or *Prohibited Method* under article 15.2, failing to submit to *Sample* collection under article 15.3, *Possession of Prohibited Substances* or *Methods* under article 15.6 or administration of a *Prohibited Substance* or *Prohibited Method* under article 15.8. If a *License-Holder* establishes in an individual case involving such violations that he bears *No Significant Fault or Negligence*, then the period of *Ineligibility* may be reduced, but the reduced period of *Ineligibility* may not be less than one-half of the minimum period of *Ineligibility* otherwise applicable. If the otherwise applicable period of *Ineligibility* is a lifetime, the reduced period under this section may be no less than 8 (eight) years. When a *Prohibited Substance* or its *Markers* or *Metabolites* is detected in a *Rider's Specimen* in violation of article 15.1 (presence of *Prohibited Substance*), the *Rider* must also establish how the *Prohibited Substance* entered his system in order to have the period of *Ineligibility* reduced.
- 266.** The Hearing Panel may also reduce the period of *Ineligibility* in an individual case where the *License-Holder* has provided substantial assistance which results in discovering or establishing an anti-doping rule violation by another *Person* involving *Possession* under article 15.6.2 (*Possession by Rider Support Personnel*), article 15.7 (*Trafficking*), or article 15.8 (administration to a *Rider*). The reduced period of *Ineligibility* may not, however, be less, in principle, than one-half of the minimum period of *Ineligibility* otherwise applicable. If the otherwise applicable period of *Ineligibility* is a lifetime, the reduced period under this Article may be no less than 8 (eight) years.
- 267.** If the sanction imposed exceeds the maximum set under these Anti-Doping Rules, it shall automatically be reduced to this maximum, without prejudice to the right of appeal of the person sanctioned.

Declaration or admission of doping

- 268.** A *License-Holder* who declares or admits to having committed an anti-doping violation shall be considered as having committed that violation on the day of the declaration or admission, unless the facts admitted or declared can be tied to a specific instance, in which case the sanctions in force at the time of the facts shall be applied.

Rules for Certain Potential Multiple Violations

- 269.** For purposes of imposing sanctions under articles 261, 262 and 263, a second anti-doping rule violation may be considered for purposes of imposing sanctions only if it is established that the *License-Holder* committed the second anti-doping rule violation after he received notice, or after a reasonable attempt was made to give notice of the first anti-doping rule violation; if not, the violations shall be considered as one single first violation, and the sanction imposed shall be based on the violation that carries the more severe sanction.
- 270.** Where a *Rider*, based on the same *Testing*, is found to have committed an anti-doping rule violation involving both a *Specified Substance* under article 262 and another *Prohibited Substance* or *Prohibited Method*, the *Rider* shall be considered to have committed a single anti-doping rule violation, but the sanction imposed shall be based on the *Prohibited Substance* or *Prohibited Method* that carries the most severe sanction.
- 271.** Where a *Rider* is found to have committed 2 (two) separate anti-doping rule violations, one involving a *Specified Substance* governed by the sanctions set forth in article 262 (*Specified Substances*) and the other involving a *Prohibited Substance* or *Prohibited Method* governed by the sanctions in article 261 or a violation governed by the sanctions in article 263.1, the period of *Ineligibility* imposed for the second violation shall be at a minimum 2 (two) years' *Ineligibility* and at a maximum 3 (three) years' *Ineligibility*. Any *Rider* found to have committed a third anti-doping rule violation involving any combination of *Specified Substances* under article 262 and any other anti-doping rule violation under article 261 or 263.1 shall receive a sanction of lifetime *Ineligibility*.
- 272.** If an anti-doping violation has been sanctioned without consideration having been given to a former condemnation for another violation, the case may be reopened at the request of the Anti-Doping Commission.
- 273.** Should an anti-doping violation be discovered that has occurred prior to another violation that has already been sanctioned, the former shall be sanctioned as a second or subsequent violation.

Disqualification of Results in Competitions Subsequent to anti-doping violation

- 274.** In addition to the automatic *Disqualification* of the results in the *Competition* pursuant to article 256, all other competitive results obtained from the date a positive *Sample* was collected (whether *In-Competition* or *Out-of-Competition*), or other doping violation occurred, through the commencement of any *Ineligibility* period, shall, unless fairness requires otherwise, be *Disqualified*.

Comment: it may be considered as unfair to disqualify the results which were not likely to have been affected by the Rider's anti-doping rule violation.

Commencement of Ineligibility Period

- 275.** The period of *Ineligibility* shall start on the date of the hearing decision providing for *Ineligibility* or, if the hearing is waived, on the date *Ineligibility* is accepted or otherwise imposed. Any period during which provisional measures pursuant to articles 217 through 223 were imposed or voluntarily accepted and any period for which subsequent *Competition* results have been *Disqualified* under article 274 shall be credited against the total period of *Ineligibility* to be served. Where required by fairness, such as delays in the hearing process or other aspects of Doping Control not attributable to the *License-Holder*, the hearing body imposing the sanction may start the period of *Ineligibility* at an earlier date commencing as early as the date of the anti-doping violation.

Reinstatement Testing

- 276.** As a condition to regaining eligibility at the end of a specified period of *Ineligibility*, a *Rider* must, during any period of provisional measures or *Ineligibility*, make himself available for *Out-of-Competition Testing* by the UCI and any other *Anti-Doping Organization* having *Testing* jurisdiction under the *Code*, and must provide current and accurate whereabouts information as provided in article 76.
- 277.** If a *Rider* subject to a period of *Ineligibility* retires from sport and is removed from UCI's *Registered Testing Pool* and later seeks reinstatement, the *Rider* shall not be eligible for reinstatement until he has notified the UCI and his National Federation and has been subject to *Out-of-Competition Testing* for a period of time equal to the longer of the period set forth in article 77 or the period of *Ineligibility* remaining as of the date the *Rider* had retired.

Consequences to teams

- 278.** Except as provided in article 279, if a *Rider* is found to have committed an anti-doping violation in connection with a team *Competition* in which he participated as a member of a team, the team shall be *disqualified* from that *Competition*.

UCI CYCLING REGULATIONS

If such *Rider* is *disqualified* from other *Competitions* of the same *Event* under articles 257.2a or 3 or article 258, any team, whether composed differently or not, of which such *Rider* was a member, shall be *disqualified* from the same *Competitions* as the *Rider*.

- 279.** In the case of a team stage during a stage race, the team shall be relegated to the last place on the stage with its real time and with a 10 (ten) minutes penalty on the general classification of teams. If more than one *Rider* on the team is found to have committed an anti-doping violation during the same team stage, the team is *disqualified* from the stage race.

XI

Chapter APPEAL TO THE CAS

- 280.** The following decisions may be appealed to the Court of Arbitration for Sport:
- a) the decisions of the hearing body of the National Federation under article 242;
 - b) a decision that a *Rider* shall be banned from participating in *Events* under article 217 if the ban is for more than 1 (one) month;
 - c) the decisions concerning Therapeutic Use Exemptions as specified under articles 67, 68, 70 and 72.
 - d) the final decision at the level of the National Federation regarding a *License-Holder* that was referred to his National Federation according to article 183.

No other form of appeal shall be permitted.

- 281.** In cases under article 280 a), the following parties shall have the right to appeal to the CAS:
- a) the *License-Holder* who is the subject of the decision being appealed;
 - b) the other party to the case in which the decision was rendered;
 - c) the UCI;
 - d) the International Olympic Committee or International Paralympic Committee, where the decision may have an effect in relation to the Olympic Games or Paralympic Games, including decisions affecting eligibility for the Olympic Games or Paralympic Games;
 - e) WADA.

- 282.** The appeal of the UCI shall be made against the *License-Holder* and against the National Federation that made the contested decision and/or the body that acted on his behalf. The National Federation or body concerned shall be liable for costs if the hearing body which made the decision against which the appeal has been made has applied the regulations incorrectly.

- 283.** An appeal by the *License-Holder* shall be made against his National Federation.

The National Federation must immediately send the UCI a copy of the statement of appeal and of any submissions or briefs made before the CAS.

The UCI shall have the right to participate in the proceedings before the CAS and demand that a sanction is imposed or increased.

- 284.** The statement of appeal by the *License-Holder* or the other party to the case must be submitted to the CAS within 1 (one) month of his receiving the full decision as specified in article 247. Failure to respect this time limit shall result in the appeal being disbarred.
- 285.** The statement of appeal by the UCI, the International Olympic Committee, the International Paralympic Committee or WADA must be submitted to the CAS within 1 (one) month of receipt of the full case file from the hearing body of the National Federation. Failure to respect this time limit shall result in the appeal being disbarred. Should the appellant not request the file within 15 (fifteen) days of receiving the full decision as specified in article 247, the time limit for appeals shall be 1 (one) month from the reception of that decision.
- 286.** If the respondent makes a counter-appeal, the appellant has the right to respond within 1 (one) month of receiving the respondent's reply, except where extended by the CAS. If the respondent is the *License-Holder*, he shall have the right to submit an additional statement within 15 (fifteen) days of receiving the appellant's reply, unless the time limit is extended by the CAS.
- 287.** In cases under article 280 b), the *Rider* only shall have the right to appeal to the CAS.
- The appeal shall be made against the UCI.
- The time to file the appeal to the CAS shall be 8 (eight) days from receipt of the decision by the *Rider* or his National Federation or his club or team.
- 288.** An appeal to the CAS shall not suspend the execution of the contested decision, without prejudice to the right to apply to the CAS for it to be suspended.
- 289.** The CAS shall have full power to review the facts and the law. The CAS may increase the sanctions that were imposed on the appellant in the contested decision.
- 290.** The CAS shall decide the dispute according to these Anti-Doping Rules and the rules of law chosen by the parties or, in the absence of such a choice, according to Swiss law.
- 291.** The decision of the CAS shall be final and binding on the parties to the case and to all *License-Holders* and National Federations. It shall not be subject to appeal.

XII

Chapter CONFIDENTIALITY AND PUBLIC DISCLOSURE

Duty of confidentiality

- 292.** Persons carrying out a task in *Doping Control* are required to observe strict confidentiality regarding any information concerning individual cases which is not required to be reported under these Anti-Doping Rules.

UCI CYCLING REGULATIONS

Such breaches of confidentiality shall be penalized by a fine of between CHF 1000 and CHF 10 000 as decided by the UCI Disciplinary Commission, which may also suspend the person in question from specified tasks for such time as it shall determine.

Public disclosure

293. Public disclosure shall be made by the Anti-Doping Commission or the National Federation as described in article 295.

294. *License-Holders* who are asserted to have committed a violation of these Anti-Doping Rules shall in principle not be publicly identified until it has been determined in a decision in accordance with articles 230 to 243 that an anti-doping violation has occurred.

However, the Anti-Doping Commission and the National Federation of the *License-Holder* who is asserted to have committed a violation of these Anti-Doping Rules may make public statements and identifications they deem appropriate under the circumstances, but not earlier than the moment of sending the notification referred to in article 224.

295. Once a violation of these Anti-Doping Rules has been established in a decision referred to in article 243, it shall be publicly reported as follows:

- if the UCI decides to appeal to the CAS, the UCI will report the violation, the decision and its decision to appeal no later than the expiration of the time limit for the appeal;
- if the UCI decides not to appeal to the CAS, it will report the violation and the decision no later than 10 (ten) days after the expiration of the time limit for the appeal.
- if the *License-Holder* or WADA appeals to the CAS, the UCI will report the violation, the decision and the appeal within 10 (ten) days after the appeal was notified to the UCI.

Publication

296. The definitive sanctions and the name of the person penalized shall be published in the UCI Official News Bulletin and/or in the official bulletin of the National Federation of the person penalized.

Register

297. The Anti-Doping Commission shall maintain a register of the penalties applied. This shall list the name of the *License-Holder*, his National Federation, his category (elite or other), the name and date of the *Event*, the penalties imposed, the date of the decision regarding the penalties and the body which imposed them.

XIII

Chapter FINAL PROVISIONS

Third Sample

- 298.** The UCI shall have the right to demand that a third *Sample* be taken at a *Sample* taking session. The Anti-Doping Commission shall issue instructions to his end to the *Anti-Doping Inspector*. The *Sample* taking procedures shall be applied *mutatis mutandis*. The taking of a third *Sample* shall be recorded.

Where appropriate, the further analysis of such *Samples* shall give rise to action for an anti-doping violation and sanctions.

Violations described under articles 15.3 and 15.5 shall apply regarding the third *Sample*.

Medication

- 299.** In *Events* designated by the Anti-Doping Commission, team or club doctors will be obliged to list all medicines taken by each *Rider* and their dosages and any medical treatment that they may have undergone during the preceding 72 (seventy-two) hours. Failure to do so shall make the team ineligible to start the race.

National Federations

- 300.** When a National Federation receives from third parties information concerning a possible anti-doping violation, it shall immediately inform the Anti-Doping Commission of the UCI.
- 301.** All National Federations shall include in their regulations the rules necessary to effectively implement these Anti-Doping Rules.
- 302.** For the purpose of these Anti-Doping Rules, the organizer's National Federation shall take on the role of the *License-Holder's* National Federation as regards *License-Holders* who have obtained their license directly from the UCI.
- 303.** Without prejudice to article 13 of the Constitution, National Federations shall be obligated to reimburse the UCI for all costs related to a doping case in which the National Federation was not cooperative or did not comply with these Anti-Doping Rules.

UCI CYCLING REGULATIONS

Non-License-Holders

- 304.** 1. If a breach of these Anti-Doping Rules is committed by a non-*License-Holder*, the Anti-Doping Commission and/or any National Federation involved shall take whatever steps are necessary to take proceedings before the competent bodies against the person in question.
2. Right to a fair hearing having granted, the Anti-Doping Commission may ban this person from attending a cycling *Event*. It may also ban any National Federation, club or trade team from making use of services offered by this person, with breaches of such a ban being subject to a fine of between CHF 1000 and CHF 10000 as determined by the Disciplinary Commission. These measures and sanctions may be taken independently of the procedure noted under paragraph 1.

Independent Observers

- 305.** Organizers shall provide access to Independent Observers as directed by the UCI.

Recognition of decisions by other organizations

- 306.** 1. Subject to the right to appeal provided in chapter XI, the *Testing*, therapeutic use exemptions and hearing results or other final adjudications of any *Signatory* to the *Code* which are consistent with the *Code* and are within the *Signatory's* authority, shall be recognized and respected by the UCI and the National Federations.
2. The UCI may recognize the same actions of other bodies which have not accepted the *Code* if the applicable rules of those bodies are otherwise consistent with the *Code*. The National Federations shall respect such actions when recognized by the UCI.
3. When agreed or otherwise decided between the UCI and the competent authorities, the UCI and the National Federations may administrate results management, hearings and appeals for the application of anti-doping legislation.

Statute of limitations

- 307.** No action may be commenced under these Anti-Doping Rules against a *License-Holder* for a violation of an anti-doping rule contained in these Anti-Doping Rules unless such action is commenced within 8 (eight) years from the date the violation occurred.

Any request for investigation or for disciplinary action and any act of investigation or disciplinary action in relation with the violation shall be considered as commencement of the action for the purpose of this article.

Interpretation of Anti-Doping Rules

- 308.** 1. The headings used for the various parts and articles of these Anti-Doping Rules are for convenience only and shall not be deemed part of the substance of these Anti-Doping Rules or to affect in any way the language of the provisions to which they refer.
2. The Introduction and the Appendix 1 "Definitions" shall be considered integral parts of these Anti-Doping Rules.
3. Notice to a *License-Holder* may be accomplished by delivery of the notice to his National Federation or as provided by these Anti-Doping Rules. The National Federation shall be responsible for making immediate contact with the *License-Holder*.
4. These Anti-Doping Rules shall not apply retrospectively to matters pending before the date these Anti-Doping Rules came into effect.

Entry into force

- 309.** This version of the Anti-Doping Rules of the UCI shall come into force on 13 August 2004, except:
- 1) Until 31 December 2004, the therapeutic use exemption rules of the Anti-Doping Examination rules in force on 12 August 2003, continue to be in force and the exemption remain valid under these Anti-Doping Rules.
- 2) Article 15.4 shall come into force on 1 January 2005.
- 3) Until the introduction of the Procedural Guidelines of the Anti-Doping Commission, *Doping Control* shall be deemed in compliance with these Anti-Doping Rules when in compliance with the Anti-Doping Examination Regulations in force on 12 August 2004.
- 4) Until 31 December 2004, results management and disciplinary proceedings (hearings and appeals), shall be deemed in compliance with these Anti-Doping Rules when in compliance with the Anti-Doping Examination Regulations in force on 12 August 2004.
- 5) The Anti-Doping Examination Regulations in force on 12 August 2004 remain in force until 31 December 2004 for *National Events*.
- 6) Any period of suspension imposed under the Anti-Doping Examination Regulations in force until 12 August 2004 ends on 12 August 2004 if at that moment it exceeds the maximum set under these Anti-Doping Rules, and is reduced to this maximum otherwise.
- 310.** Amendments to these Anti-Doping Rules shall come into force on the date of their publication in the Official News Bulletin unless this publication specifies a different date for entry into force.

(Appendix 1)

DEFINITIONS

Adverse Analytical Finding:

A report from a laboratory or other approved *Testing* entity that identifies in a *Specimen* the presence of a *Prohibited Substance* or its *Metabolites* or *Markers* (including elevated quantities of endogenous substances) or evidence of the *Use* of a *Prohibited Method*.

Anti-Doping Inspector:

Doping control officer with overall responsibility for the on site management of the *Testing* as described in these Anti-Doping Rules and in the *Procedural Guidelines*.

Anti-Doping Organization:

A *Signatory* of the Code that is responsible for adopting rules for initiating, implementing or enforcing any part of the *Doping Control* process. This includes, for example, the International Olympic Committee, the International Paralympic Committee, other *Major Event Organizations* that conduct *Testing* at their *Events*, WADA, International Federations, and *National Anti-Doping Organizations*.

Attempt:

Purposely engaging in conduct that constitutes a substantial step in a course of conduct planned to culminate in the commission of an anti-doping rule violation. Provided, however, there shall be no anti-doping rule violation based solely on an *Attempt* to commit a violation if the *Person* renounces the attempt prior to it being discovered by a third party not involved in the *Attempt*.

Code:

The World Anti-Doping Code. The Code is available on WADA's website at www.wada-ama.org or via UCI.

Competition:

A single *Race* organized separately (for example: a one day road race, each of the time trial and road race at the road World Championships) or a series of *Races* forming an organizational unit and producing a final winner and/or general classification (for example: a road race, a track sprint race tournament, a cyclo-ball tournament) (Note: a World Cup series is not an *Event* or *Competition*).

Disqualification:

See article 12.1.022 of the UCI Cycling Regulations.

Doping Control:

The process including test distribution planning, *Sample* collection and handling, laboratory analysis, results management, hearings and appeals.

Event:

A single *Competition* organized separately (for example: a one day road race, a stage race) or a series of *Competitions* conducted together as a single organization (for example: road World Championships, track World Championships, a track World Cup event); a reference to *Event* includes reference to *Competition* and *Race*, unless the context indicates otherwise.

In-Competition:

In-Competition refers to the period that starts one day before or, in the case of a major tour, three days before the day of the start of an *Event* and finishing at midnight of the day on which the *Event* finishes.

However, regarding the *Presence or Use* of a prohibited stimulant as defined in the *Prohibited List*, *In-Competition* refers to the period starting 8 (eight) hours before the start of a *Race* in which the *Rider* is competing or for which he has been confirmed as a starter and finishing at the end of the *Post-Competition Testing* session that is organized following the *Race*.

Independent Observers Program:

A team of observers, under the supervision of WADA, who observe the *Doping Control* process at certain *Events* and report on observations. If WADA is testing *In-Competition* at an *Event*, the observers shall be supervised by an independent organization.

Individual Testing:

Testing on *Riders* that is organized separately, as opposed to *Post-Competition Testing* sessions following a *Race* or *Competition*. *Individual Testing* takes place *In-Competition* (on *Riders* who participate in the *Competition* or were confirmed as starters, at any time other than during the *Post-Competition Testing* session) or *Out-of-Competition*.

Ineligibility:

Under these Anti-Doping Rules, *Ineligibility* is defined as a suspension as described in article 12.1.033 of the UCI Cycling Regulations.

Ineligibility also means that the *License-Holder* is barred, for the period of *Ineligibility*, from participating in any *Competition* or other activity organized or authorized by any *Signatory* or *Signatory's* member organization, and from any funding as provided in article 10.9 of the Code.

International Event / Competition / Race:

An *Event*, *Competition*, *Race* of the international calendar of the UCI.

International Standards:

Standards adopted by WADA in support of the Code. Compliance with the *International Standards* (as opposed to other alternative standards, practice or procedure) shall be sufficient to conclude that the procedures addressed by the *International Standards* were performed properly.

License-Holder:

- 1) A Person who is holder of a license or who has applied for a license under the UCI Cycling Regulations;
- 2) Any Person who, without being a holder of a license, participates in a cycling *Event* in any capacity whatsoever, including, without limitation, as a coach, trainer, manager, team director, team staff, agent, official, medical or para-medical personnel;
- 3) Any Person who, without being a holder of a license, participates, in the framework of a club, trade team, national federation or any other structure participating in races, in the preparation or support of *Riders* for sports competitions.

Major Event Organizations:

This term refers to the continental associations of *National Olympic Committees* and other international multi-sport organizations that function as the ruling body for any continental, regional or other international event.

Marker:

A compound, group of compounds or biological parameters that indicates the *Use* of a *Prohibited Substance* or *Prohibited Method*.

Medical Inspector:

Doping Control officer responsible for the Sample taking as described in these Anti-Doping Rules and in the *Procedural Guidelines*.

Metabolite:

Any substance produced by a biotransformation process.

Minor:

A natural Person who has not reached the age of majority as established by the applicable laws of his country of residence.

National Anti-Doping Organization:

The entity(ies) designated by each country as possessing the primary authority and responsibility to adopt and implement anti-doping rules, direct the collection of Samples, the management of test results, and the conduct of hearings, all at the national level. If this designation has not been made by the competent public authority(ies), the entity shall be the country's *National Olympic Committee* or its designee.

National Event / Competition / Race:

An *Event*, *Competition*, *Race* of the national calendar of each of the member federations of the UCI.

National Olympic Committee:

The organization recognized by the International Olympic Committee. The term *National Olympic Committee* shall also include the National Sport Confederation in those countries where the National Sport Confederation assumes typical *National Olympic Committee* responsibilities in the anti-doping area.

No Advance Notice:

A *Doping Control* which takes place with no advance warning to the *Rider* and where the *Rider* is continuously chaperoned from the moment of notification through *Sample* provision.

No Fault or Negligence:

The *Rider's* establishing that he did not know or suspect, and could not reasonably have known or suspected even with the exercise of utmost caution, that he had *Used* or been administered the *Prohibited Substance* or *Prohibited Method*.

No Significant Fault or Negligence:

The *Rider's* establishing that his fault or negligence, when viewed in the totality of the circumstances and taking into account the criteria for *No Fault or Negligence*, was not significant in relationship to the anti-doping rule violation.

Out-of-Competition:

Any *Doping Control* or fact which is not *In-Competition*.

Person:

A natural *Person* or an organization or other entity.

Possession:

The actual, physical possession, or the constructive possession (which shall be found only if the *Person* has exclusive control over the *Prohibited Substance/Method* or the premises in which a *Prohibited Substance/Method* exists); provided, however, that if the *Person* does not have exclusive control over the *Prohibited Substance/Method* or the premises in which a *Prohibited Substance/Method* exists, constructive possession shall only be found if the *Person* knew about the presence of the *Prohibited Substance/Method* and intended to exercise control over it. Provided, however, there shall be no anti-doping rule violation based solely on possession if, prior to receiving notification of any kind that the *Person* has committed an anti-doping rule violation, the *Person* has taken concrete action demonstrating that the *Person* no longer intends to have *Possession* and has renounced the *Person's* previous *Possession*.

Post-Competition Testing:

In-Competition Testing session that is organized following a *Race* or *Competition* for the purpose of testing *Riders* that participated in the *Race* or *Competition*.

Procedural Guidelines:

Documents established by the Anti-Doping Commission and regulating technical and operational parts of *Testing* pursuant to article 96; reference to these Anti-Doping Rules shall include reference to the *Procedural Guidelines* where applicable.

Prohibited List:

The List published by WADA and identifying the *Prohibited Substances* or *Prohibited Methods*.

UCI CYCLING REGULATIONS

Prohibited Method:

Any method so described on the *Prohibited List*.

Prohibited Substance:

Any substance so described on the *Prohibited List*.

Publicly Disclose or Publicly Report:

To disseminate or distribute information to the general public or persons beyond those persons entitled to earlier notification in accordance with these Anti-Doping Rules.

Race:

A cycling contest that produces a winner or a ranking according to the rules (for example: a one day road race; a stage or a half stage in a stage race, the 16th final of a track sprint race tournament, a cyclo-ball game).

Registered Testing Pool:

The pool of top level *Riders* established separately by the UCI and each National Anti-Doping Organization who are subject to both *In-Competition* and *Out-of-Competition Testing* as part of the UCI's or Organization's test distribution plan.

Rider:

A *Person* who participates as a cyclist in an *Event*, whether he is authorized to or not.

Rider's Support Personnel:

A *License-Holder* who is not a *Rider*.

Sample/Specimen:

Any biological material collected for the purposes of *Doping Control*.

Signatories:

Those entities signing the *Code* and agreeing to comply with the *Code*, including the International Olympic Committee, the UCI, International Paralympic Committee, *National Olympic Committees*, National Paralympic Committees, *Major Event Organizations*, *National Anti-Doping Organizations*, and WADA.

Specified Substance:

Prohibited Substance so identified on the *Prohibited List* pursuant to article 10.3 of the *Code*: "The *Prohibited List* may identify specified substances which are particularly susceptible to unintentional anti-doping rule violations because of their general availability in medicinal products or which are less likely to be successfully abused as doping agents".

Tampering:

Altering for an improper purpose or in an improper way; bringing improper influence to bear; interfering improperly to alter results or prevent normal procedures from occurring.

Target Testing:

Selection of *Riders* for *Testing* where specific *Riders* or groups of *Riders* are selected on a non-random basis for *Testing* at a specified time.

Testing:

The parts of the *Doping Control* process involving test distribution planning, *Sample* collection, *Sample* handling, and *Sample* transport to the laboratory.

Trafficking:

To sell, give, administer, transport, send, deliver or distribute a *Prohibited Substance* or *Prohibited Method* to any *Person* either directly or through one or more third parties, but excluding the sale or distribution (by medical personnel or by *Persons* other than a *Rider's Support Personnel*) of a *Prohibited Substance* for genuine and legal therapeutic purposes.

Use:

The application, ingestion, injection or consumption by any means whatsoever of any *Prohibited Substance* or *Prohibited Method*.

WADA:

The World Anti-Doping Agency.

(Appendix 2)

SELECTION OF RIDERS TO BE TESTED

(Article 121 of the Anti-Doping Rules)

(In the absence of specific instructions from the antidoping commission)

A. World Championships, Continental Championships, regional games

See appendix 3

B. Other events

I. ProTour (one day events)

• **General rule**

1. ProTour leader if he is present
2. The winner
3. Two riders selected at random by the inspector.

II. ProTour (stage races)

• **General rule**

1. ProTour leader after the first stage if he is present
2. ProTour leader after the last stage if he is present
3. The winner of the stage
4. The leader of the general classification after the stage
3. Two riders selected at random by the Inspector.

III. One day events (all disciplines)

• **General rule**

1. The winner
2. Two riders selected at random by the inspector.

• **World cup**

1. The winner of the race
2. The leader on the general classification of the world cup after the race
3. Two riders selected at random by the Inspector.

• **Half-stages**

1. The winner of the first half-stage
2. The winner of the second half-stage
3. One rider selected at random by the inspector for each half-stage.

• **Team events**

1. One rider selected at random by the inspector from the winning team
2. Two riders selected at random by the inspector from all other teams.

IV. Stage races (all disciplines, including prologues)

- **General rule**

1. The stage winner
2. The leader on general classification after the stage
3. Two riders selected at random by the inspector.

- **Team time trial stage**

1. One rider selected at random by the inspector from the winning team
2. The leader on general classification after the stage
3. Two riders selected at random by the inspector from all other teams.

- **Half-stages**

1. The winner of the first half-stage
2. The winner of the second half-stage
3. The leader on general classification after the second half-stage.

V. Specific time trial events

- **Individual**

1. The first three placed riders
2. Two riders selected at random by the inspector.

- **For teams**

1. One rider selected at random by the inspector from the winning team
2. One rider selected at random by the inspector from the second-placed team
3. One rider selected at random by the Inspector from each of four different randomly selected teams.

VI. Track events (all disciplines)

- **Individual**

1. The winner
2. One rider selected at random by the Inspector.

- **For teams**

1. One rider selected at random by the Inspector from the winning team
2. One rider selected at random by the Inspector from all other teams.

VII. Six-day races

1. One rider selected at random by the inspector from the winning team
2. Three riders selected at random by the inspector from three other different teams.

(text modified on 1.01.06).

(Appendix 3)**SELECTION OF RIDERS TO BE TESTED****(Article 121 of the Anti-Doping Rules)****(In the absence of specific instructions from the Anti-Doping Commission)****A. WORLD JUNIORS CHAMPIONSHIPS, CONTINENTAL CHAMPIONSHIPS, REGIONAL GAMES****1. World juniors track championships****Men**

- Kilometre time trial: first + 1 selected at random 2
- Keirin: first + 1 selected at random 2
- Sprint:
 - qualification: best time + 1 selected at random
 - final: first + 1 selected at random 4
- Team Sprint:
 - qualification: best time + 1 selected at random among the riders of the other teams
 - final: 1 rider from the first team + 1 rider selected at random among the riders of the other teams 4
- Individual pursuit:
 - qualification: best time + 1 selected at random
 - final: first + 1 selected at random 4
- Team pursuit:
 - qualification: 1 rider from the team with the best time + 1 rider selected at random among the riders of the other teams
 - final: 1 rider from the first team + 1 selected at random among the riders of the other teams 4
- Scratch: first + 1 selected at random 2
- Points race: first + 1 selected at random 2
- Madison: 1 rider from the first team + 1 rider selected at random among the riders of the other teams 2

Total men**26**

Women

| | |
|---|-------|
| • Sprint: | |
| - qualification: best time + 1 selected at random | |
| - final: first + 1 selected at random | 4 |
| • Keirin: first + 1 selected at random | 2 |
| • Individual pursuit: | |
| - qualification: best time + 1 selected at random | |
| - final: first + 1 selected at random | 4 |
| • Points race: first + 1 selected at random | 2 |
| • Scratch: first + 1 selected at random | 2 |
| • 500 m standing start time trial: first + 1 selected at random | 2 |
| | <hr/> |
| Total women | 16 |
| Total: 42 tests | |

2. World cyclo-cross championships

| | |
|----------------------------------|-------|
| Juniors | |
| - first 4 + 2 selected at random | 6 |
| Under 23 | |
| - first 4 + 2 selected at random | 6 |
| Elite | |
| - first 4 + 2 selected at random | 6 |
| Women | |
| - first 4 + 2 selected at random | 6 |
| | <hr/> |
| Total | 24 |

3. World road championships

| | |
|--|-------|
| Juniors Women | |
| • Individual - first 4 + 2 selected at random | 6 |
| • Individual time trial - first 4 + 2 selected at random | 6 |
| | <hr/> |
| Total | 12 |
| Juniors Men | |
| • Individual - 4 first + 2 selected at random | 6 |
| • Individual time trial - 4 first + 2 selected at random | 6 |
| | <hr/> |
| Total | 12 |

UCI CYCLING REGULATIONS

Under 23

- Individual - 4 first + 2 selected at random 6
- Individual time trial - 4 first + 2 selected at random 6

Total 12

Elite Women

- Individual - first 4 + 2 selected at random 6
- Individual time trial - first 4 + 2 selected at random 6

Total 12

Elite Men

- Individual - 4 first + 2 selected at random 6
- Individual time trial - 4 first + 2 selected at random 6

Total 12

Total: 60 tests

4. World track championships

Men

- Kilometre time trial: first + 1 selected at random 2
- Keirin: first + 1 selected at random 2
- Sprint:
 - qualification: best time + 1 selected at random
 - final: first + 1 selected at random 4
- Team Sprint:
 - qualification: best time + 1 selected at random among the riders of the other teams
 - final: 1 rider from the first team + 1 rider selected at random among the riders of the other teams 4
- Individual pursuit:
 - qualification: best time + 1 selected at random
 - final: first + 1 selected at random 4
- Team pursuit:
 - qualification: 1 rider from the team with the best time + 1 rider selected at random among the riders of the other teams
 - final: 1 rider from the first team + 1 selected at random among the riders of the other teams 4
- Scratch: first + 1 selected at random 2
- Points race: first + 1 selected at random 2
- Madison: 1 rider from the first team + 1 rider selected at random among the riders of the other teams 2

Total men 26

Women

- Sprint:
 - qualification: best time + 1 selected at random
 - final: first + 1 selected at random 4
- Keirin: first + 1 selected at random 2
- Individual pursuit:
 - qualification: best time + 1 selected at random
 - final: first + 1 selected at random 4
- Points race: first + 1 selected at random 2
- Scratch: first + 1 selected at random 2
- 500 m standing start time trial: first + 1 selected at random 2

Total women 16

Total: 42 tests

5. World mountain bike championships

- first 2 riders in the general classification for each category
- 1 selected at random from each category

6. World indoor cycling championships

- Artistic cycling: the champions in each discipline
- Cycle-ball: in each group A/B/C — 1 player selected at random from the 2 teams contesting the final.

7. World BMX championships

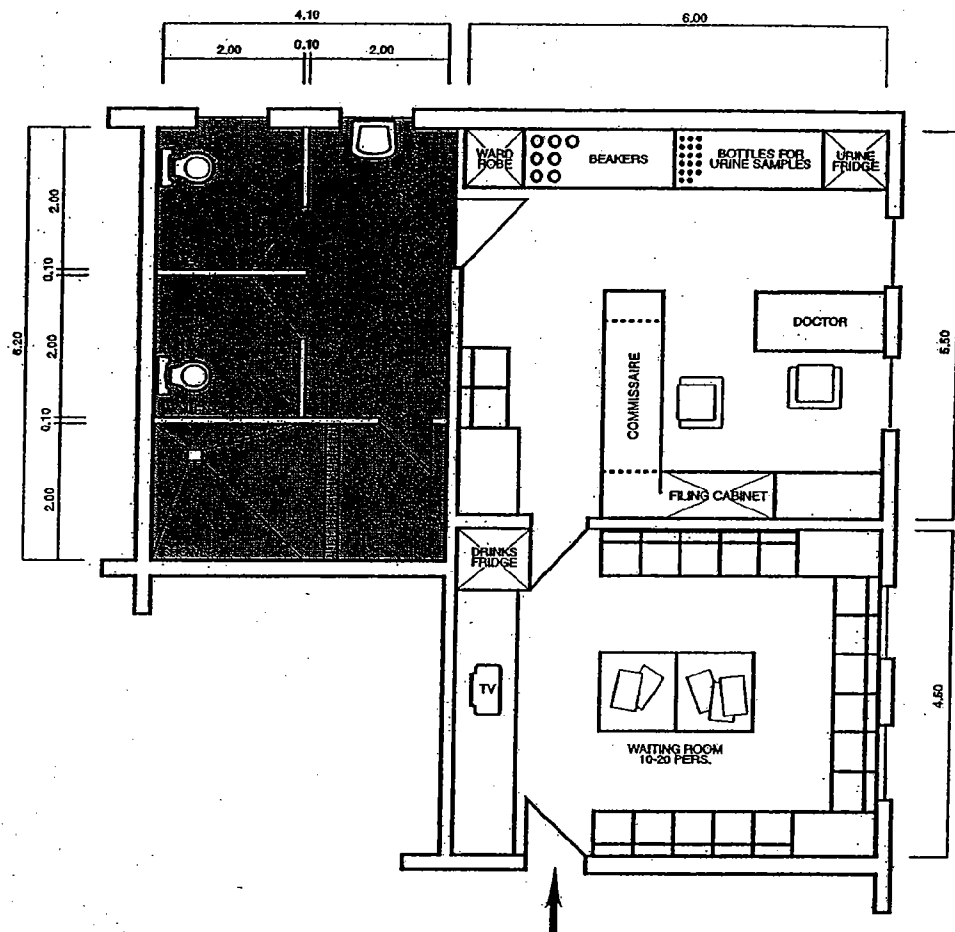
for each category

- winner
- 2 selected at random

(text modified 1.01.06).

(Appendix 4)

**PLAN OF STANDARD TESTING STATION FOR
ANTIDOPING TESTS**



(Appendix 5)

EQUIPMENT OF THE DOPING CONTROL STATION

The doping control station must be equipped in such a way to facilitate the running of the tests. A suggested list non exhaustive of equipment is included in mentioned hereafter:

Equipment to be made available:

- Anti-doping kits*
- containers for the taking
- gloves
- provisional seal kits
- plastic bags in reserve
- packaging to send the kits
- adhesive tape
- envelopes
- string
- scissors
- drinks in large quantities (such as lemonade, mineral water, etc.)

* in sufficient number to allow the choice between two kits at least.

Furnishings

First room
(20-25 m²)

- 2 tables
- 8 chairs
- 1 refrigerator
- 1 waste bin
- 1 telephone connection (phone and fax).

Second room

- 2 tables
- 3 chairs
- toilet (WC)
- sink, shower
- 1 waste bin
- towels and soap
- ventilation or opening window.

(text modified on 1.01.06).

(Appendix 6)

On chemical self-copying paper in triplicate (1 original and 2 copies).
Original to UCI - Copy 1 (white) to the rider - Copy 2 (green) to the laboratory.
(articles 163 to 165 of the Anti-Doping Rules)

Union Cycliste Internationale ANTIDOPING CONTROL

Test certificate

| | | | |
|---|---------------------------|---------------------------|--------------------------|
| Urine test | <input type="radio"/> | Blood test | <input type="radio"/> |
| 1. In competition | <input type="radio"/> | 2. Sex: Male | <input type="radio"/> |
| Hors-compétition | <input type="radio"/> | Female | <input type="radio"/> |
| 3. Date: | | 4. Place | |
| 5. Race (name, stage): | | 6. Discipline: | |
| 7. Surname of the rider: | | First name: | |
| Address of the rider: | | | |
| 8. UCI Code: | | | |
| 9. National federation which delivered the license | | | |
| 10. License number: | | | |
| 11. Random | Yes <input type="radio"/> | No | <input type="radio"/> |
| 12. Presentation time: | | | |
| 13. Time of sampling: | | | |
| Volume pH* | | Specific gravity | |
| * Optional | | | |
| 14. Bottle codes: | | | |
| 15. Time of rider's refusal: | | | |
| 16. Pharmaceutical drugs taken by the rider: | | | |
| Contents of the health booklet: | | | |
| 17. Therapeutic use exemption (TUE) | | Yes <input type="radio"/> | No <input type="radio"/> |
| 18. Subject to the comments below, I confirm that the sample was taken in accordance with the regulations. | | | |
| Rider's signature who also acknowledges receipt of his copy: | | | |
| 19. Assistant: | | | |
| name | | signature | |
| 20. Examining doctor: | | | |
| name | | signature | |
| Blood collection officer: | | | |
| name | | signature | |
| 21. Antidoping inspector: | | | |
| name | | signature | |

(Appendix 7)

NOTIFICATION TO THE RIDER
(articles 138 to 144 of the Anti-Doping Rules)

Where applicable,
Name of the race

The rider
Name of the rider

Phone

UCI Code

National licence number

National federation which delivered the licence

is required to attend an anti-doping test (urine ☐ blood ☐) at (time)
precisely at the following location:

If the rider does not attend the test, a violation of the UCI Anti-Doping Rules will be noticed and the rider could be sanctioned in accordance with Chapter X of these Rules.

Refusal (reasons)

This notification was issued

Place

Date

Time

Signature for receipt

The rider

and/or

The team leader/team manager

Name

Signature:

Anti-doping Inspector

Name

Signature

(text modified on 1.01.06).

(Appendix 8)

NO-SHOW REPORT
(articles 15.3 and 257 of the Anti-Doping Rules)

To be sent to the UCI

I the undersigned
appointed to officiate as Anti-Doping Inspector for the event
on at

do hereby certify that:

Rider No., who was properly designated to undergo the anti-doping tests and notified by all the means made available to me by the organiser, did not attend at the designated testing station within the deadline that conforms to the regulations.

- Time of arrival at the finish line
- End of the official ceremony
- Time when the rider's deadline was observed to have expired

In consequence, the present no-show report has been issued in his/her regard.

Done in, at
Signature of the Anti-Doping Inspector

for nomination¹⁾

- m Placingat the finish
- m Drawn by lot
- m Reserve
- m Instructions of the Anti-Doping Commission

Means used to notify the rider¹⁾

- m Posted at the finish line
- m Radio announcement to
- m Posted on the door of the testing station
- m Written summons issued
- m Others

Distance from finish line to the testing station

Further information on the rider

Name

First name

Nationality

National licence number

UCI Code

¹⁾ Check as appropriate
(text modified on 1.01.06).

(Appendix 9)

NOTIFICATION TO THE RIDER OF A POSITIVE RESULT
(article 212 of the Anti-Doping Rules)

Name of the race
The rider
• name
• UCI Code
• national licence number
• national federation which delivered the license

is hereby informed that he/she was found to have tested positive after the following

Name of the stage
Date of the stage
or name of the six-day event
Date of testing
The analysis carried out at the following laboratory
Name of laboratory
Full address
has shown the presence of
Name of the substances or methods

The rider has been questioned. He/she has received the form to request for a counter-analysis.

Please note:

- 1) The rider has the right to request counter-analysis;
- 2) Such a request must be submitted to the inspector within 3 hours of receiving the present notification specified in article 212;
- 3) If not, the rider shall automatically be disqualified.

Done on:

Date Place Time

By:

Name the president of the commissaires panel

Signature

Rider's comments

Rider's signature

Name and signature of the rider's assistant

(text modified on 1.01.06).

(Appendix 10)

REQUEST FOR COUNTER-ANALYSIS
(articles 212 to 216 of the Anti-Doping Rules)

(to be submitted to the Inspector within 3 hours of notification of the positive result)

Name of the race
The undersigned
Name and surname of the rider
UCI Code
National licence number
National federation which delivered the license

requests a counter-analysis in connection with the positive result of the anti-doping tests

Name and date of the stage for which the result was positive
Date of anti-doping test (six-day events)

Done on:

Place
Date
Time
Signature of rider

Request received at

Place
Date
Time

by:

Name of the Anti-Doping Inspector
Signature

Copy of the request received by

Name
Signature

(text modified on 1.01.06).

UCI CYCLING REGULATIONS

(Appendix 11)

LIST OF MEDICINES TAKEN (article 299 of the Anti-Doping Rules)

Name of the race..... Country.....
Date of the race.....
Team/Club.....
The undersigned team/club doctor.....
Name and address.....

declare that in the 72 hours prior to the start of the event the following riders* have taken medicines or undergone treatment as follows:

| Rider | Medicine or treatment (indicate dose and manufacturer) |
|----------|---|
| 1. | |
| 2. | |
| 3. | |
| 4. | |
| 5. | |
| 6. | |
| 7. | |
| 8. | |
| 9. | |
| 10. | |

Date.....
Signature.....

* N.B. - All the riders of the team/club taking part in the event must be listed; where applicable indicate «none».

(Appendix 12)

CODE OF SPORTS-RELATED ARBITRATION (CAS)
(available on request)

CAS 2004/O/645

AWARD

rendered by

THE COURT OF ARBITRATION FOR SPORT

sitting in the following composition:

President : L. Yves **Fortier**, CC, QC, Barrister in Montreal, Canada
Arbitrators : Christopher L. **Campbell**, Esq., Attorney-at-law in Fairfax, United States
Peter **Leaver**, QC, Barrister in London, United Kingdom

Ad hoc Clerk : Stephen L. **Drymer**, Attorney-at-law in Montreal, Canada

In the arbitration between:

UNITED STATES ANTI-DOPING AGENCY

Claimant

Represented by Travis T. Tygart, Esq., Director of Legal Affairs, *United States Anti-Doping Agency*, and by Richard R. Young, Esq. and Matthew S. Barnett, Esq., of the law firm *Holme Roberts & Owen, LLP*.

- and -

TIM MONTGOMERY

Respondent

TABLE OF CONTENTS

| | |
|---|------------------------------------|
| I. INTRODUCTION | 1 |
| II. THE PARTIES | 3 |
| III. PROCEDURAL BACKGROUND..... | 4 |
| A. USADA's "Charging Letter" | 4 |
| B. The Decision to Proceed Directly to CAS..... | 7 |
| C. Commencement of the Arbitration and Constitution of the Panel..... | 7 |
| D. Initial Stage of the Proceedings and the CAS Order of Procedure (August - October 2004)..... | 8 |
| E. First Preliminary Hearing: Procedural Timetable and Related Issues (1 November 2004) | 10 |
| F. Second Preliminary Hearing: Jurisdiction of the Panel (15 December 2004)..... | 11 |
| G. Third Preliminary Hearing: Evidentiary Issues and Objections (21 February 2005)..... | 11 |
| H. The Hearing on the Merits (6 - 10 June 2005)..... | 14 |
| IV. THE CASE AGAINST MR. MONTGOMERY | 15 |
| I. Applicable IAAF Rules | 15 |
| J. USADA's 7 Types of Evidence | 16 |
| K. Kelli White's Testimony..... | 17 |
| L. Mr. Montgomery's Decision Not to Testify | 18 |
| V. DECISION | 20 |
| M. The Doping Offence | 20 |
| N. The Sanction..... | 21 |
| VI. CONCLUSION..... | 22 |
| VII. COSTS | 23 |
| VIII. PUBLICATION OF THE AWARD | ERREUR ! SIGNET NON DEFINI. |

I. INTRODUCTION

1. This Award is the culmination of an exhaustive process of briefings and hearings, discussions amongst the parties, and numerous interventions by the Panel.

2. At issue is the charge by the United States Anti-Doping Agency ("USADA") that Tim Montgomery violated applicable IAAF anti-doping rules, notwithstanding that Mr. Montgomery never tested positive in any in-competition or out-of-competition drug test. As such, the issues raised in this so-called "non-analytical positive" case are, if not wholly novel, certainly not in the nature of issues arising in a typical "adverse analytical finding" (or "analytical positive") doping case. However, as explained more fully below, and to quote the Panel in the case of *USADA v. Michelle Collins* (another non-analytical positive case that arose in similar circumstances), "the straightforward application of legal principles to essentially undisputed facts leads to a clear resolution of this matter."

3. USADA seeks a four-year sanction of Tim Montgomery for participating in a wide-ranging doping conspiracy implemented by the Bay Area Laboratory Cooperative ("BALCO"). USADA charges that, for a period of several years, Mr. Montgomery used various performance-enhancing drugs provided by BALCO. As noted, Mr. Montgomery has never had a single drug test found to be a positive doping violation, but USADA's charges are based, in part, on all of the blood and urine tests at IOC-accredited and non-IOC-accredited laboratories that he has had in recent years. USADA also relies, among other things, on documents seized by the U.S. government from BALCO that have been provided to USADA; statements made by BALCO officials; and other documents.

4. According to USADA, BALCO was involved in a conspiracy the purpose of which was the distribution and use of doping substances and techniques that were either undetectable or difficult to detect in routine drug testing. BALCO is alleged to have distributed several types of banned doping agents to professional athletes in track and field, baseball and football. Among these were tetrahydrogestrinone ("THG"), otherwise known as "the Clear" by BALCO and its users. THG is a designer steroid that could not be identified by routine anti-doping testing until 2003, when a track and field coach provided a sample of it to USADA. It is undisputed that the Clear is a prohibited substance under the IAAF Rules.

5. On 3 September 2003, FBI agents searched BALCO's premises pursuant to search warrants. Approximately twenty-four agents searched BALCO's offices and seized hundreds of documents there and at other locations maintained by BALCO. The agents also seized samples of the Clear and other substances distributed by BALCO. During this raid, agents interviewed the company's President, Victor Conte, and other BALCO officials, who spoke about its activities and its customers. Mr. Conte named fifteen track and field athletes whom he alleged were clients of BALCO, including Mr. Montgomery, as well as other athletes from the NFL and Major League Baseball.

6. Following the BALCO raid, government agents obtained other documents, such as emails, through the use of subpoenas and other law enforcement mechanisms. Additional records were produced and created as part of the Grand Jury investigation, which resulted in the indictment of Mr. Conte, along with several alleged co-conspirators.¹ None of the evidence in this case derives from the Grand Jury proceedings. However, the BALCO documents were obtained by the U.S. Senate, which subsequently provided them to USADA.

7. As will be seen, the Panel's determination of the case against Mr. Montgomery turns on certain statements made by the Respondent himself which make it unnecessary for the Panel to determine whether the mass of other evidence adduced by USADA and derived in large measure from the BALCO documents, is also conclusive of the doping charges brought against him.

8. This Award is also the culmination of a process which saw two separate cases run essentially in parallel. Although the facts alleged in the present case and in the case of *USADA v. Chryste Gaines* differed in their detail, and separate submissions were filed by the parties in each case, both the nature of the charges brought against the Respondents and the substantive and procedural positions adopted by them throughout the period leading up to their respective hearings were so similar as to be virtually indistinguishable. Among other consequences, this meant that, but for the hearings on the merits, and with the consent of all parties, the two cases proceeded in lockstep. This is immediately apparent from a reading of the two Awards, which

¹ Mr. Conte pleaded guilty to several of the charges against him and, in October 2005, was sentenced to four months in prison plus four months of home confinement.

are being rendered simultaneously by the Panels (composed of the same arbitrators) in the two cases.

II. THE PARTIES

9. The Claimant, USADA, is the independent Anti-Doping Agency for Olympic sports in the United States and is responsible for managing the testing and adjudication process for doping control in that country. In that capacity, USADA conducts drug testing and results management for participants in the Olympic movement within the United States.²

10. The Respondent, Tim Montgomery ("**Mr. Montgomery**" or the "**Athlete**"), is an elite and highly successful American track and field athlete. As a sprinter, Mr. Montgomery has won numerous track and field titles, including World Championship and Olympic gold medals, as well as a world record.³

11. On 17 September 2004, The International Association of Athletics Federations (the "**IAAF**"), the international federation responsible for the sport of athletics worldwide, requested permission to appear in the arbitration as a party (i.e., as an intervener). In its request, the IAAF stated that, under IAAF Rules, should the Panel allow it to appear as a party, the Panel's award "... will be final and binding and no further reference may be made to the CAS" and, further, that "the IAAF is content for this to be the final decision on [the Athlete's] eligibility." The IAAF's request was granted by the Panel on 4 October 2004. On 22 October 2004, the IAAF specified that the sole issue in respect of which it might make submissions in the arbitration concerned "the position that may be adopted by the parties in relation to IAAF Rules and their proper construction." The Panel subsequently declared that "... the IAAF participating in [this case] as described above, the [award] rendered by the Panel shall be final and binding on the IAAF, without possibility of appeal."⁴ In the event, after

² See: United States Anti-Doping Agency Protocol for Olympic Movement Testing (effective 7 October 2002) (the "USADA Protocol"), Mission Statement and para. 1, Exhibit A to USADA's Request for Arbitration dated 5 July 2004.

³ The Panel notes that by letter dated 1 December 2005, counsel of record throughout the arbitration, Mr. Howard L. Jacobs and Ms. Jill A. Benjamin of the law firm *Forgey & Hurell, LLP*, and Ms. Cristina Arguedas and Ms. Julie Salomon of the law firm *Arguedas, Cassman & Headley*, notified the CAS that they were withdrawing as Mr. Montgomery's counsel in these proceedings.

⁴ All of this is described in detail in the Panel's 9 November 2004 correspondence to the parties, discussed further below.

considering the written submissions filed by Claimant and Respondent, the IAAF notified the CAS that it did not intend to make any independent submissions in the arbitration.

12. With the consent of Claimant and Respondent and of the Panel, several third parties were also granted permission by CAS to attend the hearings as "observers". These were: a representative of the World Anti-Doping Agency ("WADA"); John Ruger, the United States Olympic Committee ("USOC") Athlete Ombudsman; and a member of the staff of U.S. Congressman John Conyers, Jr. At the end of the day, Congressman Conyers chose not to send a representative to any of the hearings, WADA attended only the two preliminary hearings held on 15 December 2004 and 21 February 2005, and Mr. Ruger was the sole observer at the hearing on the merits.

III. PROCEDURAL BACKGROUND

A. USADA's "Charging Letter"

13. On 7 June 2004, USADA informed Respondent that it had received evidence which indicated that Mr. Montgomery was a participant in a doping conspiracy involving various elite athletes and coaches as well as BALCO. On the same date, USADA submitted the matter to its Anti-Doping Review Board (the "**Review Board**") pursuant to paragraph 9 (a) (i) of the USADA Protocol. In accordance with the provisions of that paragraph, the Athlete also submitted a lengthy and detailed submission on the matter to the Review Board.

14. By letter dated 22 June 2004 (the so-called "**Charging Letter**"), USADA informed Mr. Montgomery that, after consideration of the documents submitted to it by USADA and Mr. Montgomery, and in accordance with paragraph 9 (a) (i) (vi) of the USADA Protocol, the Review Board had determined that there existed "sufficient evidence against you to proceed with the adjudication process as set forth in [the USADA Protocol]."⁵ The charges against Respondent were set out in the Charging Letter, and reiterated in USADA's Statement of Claim, as follows:

⁵ Para 9 (a) (i) (vi) of the USADA Protocol reads as follows: "The Review Board shall consider the written information submitted to it and shall, by majority vote, make a recommendation to USADA with a copy to the Athlete whether or not there is sufficient evidence of doping to proceed with the adjudication process."

[A]t this time, and reserving all rights to amend the charge, USADA charges you with violations of the IAAF Anti-Doping Rules. (...) USADA charges that your participation in the Bay Area Laboratory Cooperative ("BALCO") conspiracy, the purpose of which was to trade in doping substances and techniques that were either undetectable or difficult to detect in routine testing, involved your violations of the following IAAF Rules that strictly forbid doping⁶:

- Rule 55.2

The offence of doping takes place when either:

- (i) a prohibited substance is present within an athlete's body tissues or fluids; or
- (ii) an athlete uses or takes advantage of a prohibited technique; or
- (iii) an athlete admits having used or taken advantage of a prohibited substance or a prohibited technique (See also Rule 56).

- Rule 56.3

Any person assisting or inciting others, or admitting having incited or assisted others, to use a prohibited substance, or prohibited techniques, shall have committed a doping offence and shall be subject to sanctions in accordance with Rule 60. If that person is not an athlete, then the Council may, at its discretion, impose an appropriate sanction.

- Rule 56.4

Any person trading, trafficking, distributing or selling any prohibited substance otherwise than in the normal course of a recognised profession or trade shall also have committed a doping offence under these Rules and shall be subject to sanctions in accordance with Rule 60.

- Rule 60.1

For the purpose of these Rules, the following shall be regarded as "doping offences" (see also Rule 55.2):

- (i) the presence in an athlete's body tissues or fluids of a prohibited substance;
- (ii) the use or taking advantage of forbidden techniques;

⁶ The text quoted is from the 2002 IAAF Rules. The version of the rules released in 2000 includes the following variations in language: Rule 60(1)(i) requires 'the finding in an athlete's body' [as opposed to 'the presence in an athlete's body tissues or fluids']; and Rule 60(1)(iii) excludes the phrase 'or having attempted to use.'

(iii) admitting having taken advantage of, or having used, or having attempted to use, a prohibited substance or a prohibited technique;

(...)

(vi) assisting or inciting others to use a prohibited substance or prohibited technique, or admitting having admitted or incited others;

(vii) trading, trafficking, distributing or selling any prohibited substance.

Specifically, the evidence confirms your involvement with the following prohibited substances and prohibited techniques: one or more substances belonging to the prohibited class of "Anabolic Steroids;" Testosterone/Epitestosterone Cream; EPO; Growth Hormone; and Insulin.

15. As regards the sanction for these alleged violations USADA stated as follows in its Charging Letter:

USADA applies the sanctions found in the rules of the relevant International Federations and the USOC Anti-Doping Policies. Therefore, at this time reserving all rights to amend the sanction at a later date, under the Rules of the IAAF, Division III, Rule 60, USADA is seeking the following sanction against you for your doping offense:

- A lifetime period of ineligibility beginning on the date you accept this sanction or the date of the hearing panel's decision;⁷
- The retroactive cancellation of all awards or additions to your trust fund to which you would have been entitled by virtue of your appearance and/or performance at any athletics meeting occurring between February 1, 2000 and the date your period of ineligibility begins, pursuant to Division III, Rule 60.5 of the IAAF Anti-Doping Rules; and,
- A lifetime period of ineligibility beginning on the date you accept this sanction or the date of a hearing panel's decision, from participating in a US Olympic, Pan American Games or Paralympic Games, trials or qualifying events, being a member of any US Olympic, Pan American Games or Paralympic Games team and having access to the training facilities of the United States Olympic Committee ("USOC") Training Centers or other programs and activities of the USOC including, but not limited to, grants, awards or employment pursuant to the USOC Anti-Doping Policies.

⁷ At the final hearing, by which time certain of the charges (in particular the charge of "trafficking") against the Respondent had been dropped, USADA requested that the Panel impose a *four-year* period of ineligibility on Mr. Montgomery.

B. The Decision to Proceed Directly to CAS

16. In response to the Charging Letter, Mr. Montgomery notified USADA on 28 June 2004 that, in conformity with paragraph 9 (b) (iv) of the USADA Protocol, he elected to "bypass the domestic hearing process" described in paragraph 9 (b) (ii) of the USADA Protocol and "proceed directly to a single final hearing before the Court of Arbitration for Sport."⁸

17. The Respondent did not state in his letter, though the Panel considers it significant to note, that paragraph 9 (b) (iv) of the USADA Protocol provides that upon an athlete making such an election, "[t]he CAS decision shall be final and binding on all parties and shall not be subject to further review or appeal."

C. Commencement of the Arbitration and Constitution of the Panel

18. On 5 July 2004, Claimant submitted its Request for Arbitration to the CAS. The Request for Arbitration substantially reprised the allegations set out in USADA's 22 July 2004 Charging Letter, and identified Peter Leaver, QC, barrister, of London, England, as USADA's party-appointed arbitrator.

19. The Request for Arbitration also noted the parties' agreement that, in the event that Mr. Montgomery were to qualify for the US Olympic team for the 2004 Summer Games in Athens the following month, the arbitration proceedings would be expedited.⁹

20. Mr. Montgomery submitted his Answer to USADA's Request for Arbitration on 6 July 2004. In his Answer, the Athlete provided a brief statement of his defence and named Christopher L. Campbell, Esq., attorney-at-law, of San Francisco, U.S.A, as his party-appointed arbitrator.

21. The two party-appointed arbitrators subsequently selected L. Yves Fortier, CC, QC, barrister and solicitor, of Montréal, Canada, to serve as President of the Panel.¹⁰

⁸ Mr. Montgomery's letter of 28 June 2004 is filed as Exhibit B to USADA's Request for Arbitration.

⁹ In the event, Mr. Montgomery did not qualify for the US Olympic team.

¹⁰ The constitution of the Panel was formally notified to the parties by means of an "Order of Procedure" issued by the CAS on 8 September 2004. See below.

22. In due course, the CAS appointed Stephen L. Drymer, barrister and solicitor, of Montréal, Canada, to assist the Panel in the capacity of *ad hoc* clerk.

D. Initial Stage of the Proceedings and the CAS Order of Procedure (August - October 2004)

23. Far from "expediting" matters, as might originally have been their intention, the parties instead proved unable, during the initial stage of the arbitration, to collaborate with each other and the Panel as required to speed matters along. Having observed as much, the Panel acknowledges the unique and complex nature of the issues raised in this case, which no doubt meant that additional time was required for the parties to elucidate (let alone for the Tribunal to determine) the numerous substantive and procedural issues which arose in the course of the proceedings.

24. On 8 September 2004, the CAS issued its standard "Order of Procedure" addressing such matters as the jurisdiction of the CAS, the composition of the Panel, provisions regarding the costs of the arbitration and a statement concerning the confidentiality of the proceedings. The Order of Procedure also established a timetable for the filing of written submissions by the parties in accordance with article R44.1 of the CAS Code of Sports-related Arbitration (the "CAS Code"), leading to a hearing on the merits in San Francisco during the week of 1-5 November 2004. (As discussed below, that timetable quickly proved to be unfeasible and was in due course modified.)

25. The 8 September 2004 Order of Procedure further confirmed that the conduct of the arbitration was governed by articles R38 and following of the CAS Code, that is, by the CAS rules applicable to "Ordinary" (first instance) arbitrations as opposed to "Appeal" arbitrations.

26. The parties subsequently filed their respective written submissions – a Statement of Claim and a Response, together with supporting evidence – as required by the Order of Procedure.

27. As indicated above, the period leading to the planned 1 November 2004 hearing was characterized by an acrimonious flurry of correspondence, requests, objections, accusations and counter-accusations, motions and applications, the overall effect of which ultimately led both parties to request that the November 2004 hearing dates be vacated.

28. Among the procedural decisions and orders that the Panel was called upon to render during this period, several deserve mention.

- On 20 September 2004, the Panel denied two motions brought by USADA, one to compel the giving of consent by Mr. Montgomery for USADA to access certain medical records, and the second to compel Mr. Montgomery to answer certain "requests for admissions". The Panel also addressed a motion by USADA to issue subpoenas to various individuals. In this latter regard, the Panel agreed with USADA's submission that it has the power to issue subpoenas enforceable by United States courts; however, it requested the parties to provide additional briefing concerning the form of such subpoenas taking into account the provisions of article R44.3 of the CAS Code as well as Article 7 of the U.S. *Federal Arbitration Act* and Rule 45 of the U.S. *Federal Rules of Civil Procedure*.
- On 26 September 2004, the Panel signed and issued a "Stipulated Protective Order" negotiated by the parties governing the disclosure of confidential information by USADA to Respondent.
- On 7 October 2004, having considered the parties' submissions on the matter of the subpoenas requested by USADA, the Panel issued subpoenas to five individuals compelling their attendance (and in certain cases requiring the production of specified documents by them) at the 1 November hearing.¹¹
- On 19 October 2004, the Panel denied the parties' request (originally formulated by Respondent and consented to by Claimant), that the hearing on the merits be postponed from 1 November to a date "to be determined." The Panel instead reconfirmed that the hearing would commence on 1 November 2004. The Panel informed the parties that the first issue to be addressed at that hearing would be the determination of an appropriate and detailed schedule for the presentation of the parties' evidence; subsequently, the Panel would receive documents from those witnesses to whom subpoenas will have been issued and thereafter, and subject to any

¹¹ The Panel denied USADA's requests for subpoenas to be issued to various reporters as well as to the Respondent himself.

determinations made with respect to a detailed hearing schedule, the evidentiary phase of the hearing would commence, it is being understood that additional hearing days would also be scheduled.

- On 20 October 2004, the Panel responded to Mr. Montgomery's motion for issuance of subpoenas dated 15 September 2004. As in the case of the subpoenas previously requested by USADA, the Panel granted the Athlete's request by issuing subpoenas to several individuals compelling their attendance and requiring the production of documents by them at the hearing set to commence on 1 November 2004.

29. One further occurrence during this period deserves mention. On 26 October 2004, the parties entered into a "joint stipulation" in which they noted the existence of numerous disagreements regarding "threshold procedural and evidentiary issues, the resolution of which are fundamental to determining the most efficient presentation of [this case]" and acknowledged their inability "to reach any agreement on these procedural and evidentiary issues that would facilitate the orderly and efficient presentation of [this case]." The stipulation further recorded the parties' agreement to vacate the hearing dates during the week of 1 November as well as their agreement regarding a (partial) procedural timetable leading to a hearing to be scheduled at an undetermined date in 2005. The Panel responded to this development by a letter dated 28 October 2004. The Panel expressed its "surprise at this last minute development." It informed the parties that, in the circumstances, the hearing on the merits clearly could not commence on 1 November, yet it nonetheless ordered the parties' legal representatives to meet with the Panel in San Francisco on 1 November "in order to discuss fully all outstanding procedural and evidentiary issues and seek to determine a reasonable calendar for the future conduct of [this arbitration]."

E. First Preliminary Hearing: Procedural Timetable and Related Issues (1 November 2004)

30. A preliminary hearing was accordingly held in San Francisco on 1 November 2004. The outcome of that hearing is described in detail in a letter to the parties dated 9 November

2004, in which the Panel confirmed a series of procedural orders issued orally during the hearing itself.¹²

31. As noted in the Panel's 9 November letter, the hearing "was considered necessary by the Panel in view of what it believed to be insufficient progress made by the parties themselves – as illustrated, for example, in their Joint Stipulation of 26 October 2004 – in establishing a clear timetable for the fair and efficient determination of [this case]." The procedural orders issued on 1 November, and confirmed in writing on 9 November, addressed a series of issues ranging from the re-issuance of subpoenas previously issued at the request of the parties,¹³ the identity of the individuals authorized to participate in the arbitration as observers or interveners, as well as, most importantly, a list of outstanding procedural and evidentiary issues raised by the parties and a detailed timetable for the briefing and hearing of those issues.

F. Second Preliminary Hearing: Jurisdiction of the Panel (15 December 2004)

32. In accordance with the timetable established on 1 November 2004 and confirmed in writing on 9 November, a preliminary hearing was held, in Montreal, on 15 December 2004 on the matter of a Motion brought by the Athlete to dismiss the case on the ground that the CAS lacked jurisdiction.

33. The nature of the parties' submissions and the positions taken by them both in writing and at the hearing are described in the Panel's Award on Jurisdiction dated 9 February 2005. For present purposes it suffices to note that, for the reasons set out in that Award, the Panel dismissed Respondent's Motion and affirmed its jurisdiction in this matter.

G. Third Preliminary Hearing: Evidentiary Issues and Objections (21 February 2005).

34. A further, and final, preliminary hearing was held, in Montreal, on 21 February 2005 for the purpose of hearing the parties' submissions on a variety of evidentiary issues and objections raised by Respondent. Once again, reference is made to the detailed Decision on

¹² It is noted that a court reporter was engaged to record the proceedings of the 1 November 2004 hearing, and that a transcript of those proceedings was provided to the parties and to the CAS.

¹³ None of the individuals to whom subpoenas had been issued in fact appeared at the 1 November hearing and neither party produced any of the documents requested of these individuals.

Evidentiary and Procedural Issues rendered by the Panel on 4 March 2005 in respect of the matters addressed at that hearing.

35. With the Panel's 4 March 2005 Decision, the nature of the allegations against the Respondent was clarified, certain additional submissions were requested of the parties and, in short, the path toward the hearing on the merits was cleared.

36. The Panel's Decision on Evidentiary and Procedural Issues also addressed the question of the standard of proof applicable in the present case, which had been in dispute as between the parties. In view of the importance of the issue the Panel considers it apposite to reproduce the relevant passages of its 4 March 2005 Decision, which are as follows:

Standard of Proof

There is no dispute as to which of the parties, whether Claimant or Respondents, bears the onus of establishing the charges that have been levelled against Mr. Montgomery and Ms. Gaines in these cases. All parties accept that USADA bears the burden of proof in respect of its claims.

There is no such common understanding, however, in respect of the standard of the proof to be made by USADA in order for it to succeed – that is, whether USADA must prove its claims beyond reasonable doubt, as advocated by Respondents, or whether it need only make proof on the balance of probability.

The athletes' submissions are based on the argument (to quote from Mr. Montgomery's Motion on Burden of Proof, at p. 2) that "the U.S. Supreme Court has held that the burden of proof is a substantive rule [that cannot be applied retroactively]," and on the fact that "[p]rior to March 2004, IAAF Rule 59.6 provided that in all doping hearings, 'the Member shall have the burden of proving, beyond reasonable doubt, that a doping offense has been committed'." As further summarised by the athletes' counsel during the 21-22 February 2005 hearing, given that "that is what the new Rules say, you don't even have to consider the substantive/procedural issue."

As set out in its Statements of Claim, USADA's claims against the athletes for violations of IAAF Rules concern allegations that Respondents engaged in systematic doping "commencing in February 2000" (in Mr. Montgomery's case) and "commencing in September 2000" (as regards Ms. Gaines); and, as noted above, USADA refers specifically to alleged violations of the 2002 IAAF Rules. As of 1 March 2004, the IAAF implemented the provisions of the World Anti-Doping Code in new IAAF Anti-Doping Rules, including the provision (Article 3.1 of the World Anti-Doping Code: "Burdens and Standards of Proof") that "[t]he standard of proof shall be whether the Anti-Doping Organization has established an anti-doping rule violation *to the comfortable satisfaction of the hearing body, bearing mind the seriousness of the allegation which is made.*" (Emphasis added)

USADA, not surprisingly, sees things differently than the Respondents. It acknowledges (at p. 42 of its 9 February 2005 Response Brief) that what it calls “[t]he old ‘beyond reasonable doubt’ standard” was replaced by the IAAF as of 1 March 2004. The crux of USADA’s argument is that “[t]he introduction to the new IAAF Rules state that the new rules ‘shall not be applied retrospectively to doping matters pending at 1 March 2004’; *by negative implication, this introductory statement suggests that the new rules may be applied to doping charges initiated after March 1, 2004.*” (Emphasis added) USADA goes on to challenge the Respondents’ view that the standard of proof is a substantive, as opposed to a procedural, rule; and it refers to U.S. case law as well as CAS precedent in support of the principle that the criminal law standard of proof is inapplicable to these proceedings.

As often becomes evident when the question of standard of proof is debated, the debate looms larger in theory than practice. Counsel for all parties concurred with the views expressed by the members of the Panel during the 21-22 February 2005 hearing to the effect that even if the so-called “lesser”, “civil” standard were to apply – namely, proof on the balance of probability, or, in the specific context in which these cases arise, proof to the *comfortable satisfaction* of the Panel *bearing mind the seriousness of the allegation which is made* (what might be called the “comfortable satisfaction” standard) – an extremely high level of proof would be required to “comfortably satisfy” the Panel that Respondents were guilty of the serious conduct of which they stand accused.

Even under the traditional civil model, there is no absolute standard of proof. Built into the balance of probability standard is a generous degree of flexibility that relates to the seriousness of the allegations to be determined. In all cases the degree of probability must be commensurate with and proportionate to those allegations; the more serious the allegation the higher the degree of probability, or “comfort”, required. That is because, in general, the more serious the allegation the less likely it is that the alleged event occurred and, hence, the stronger the evidence required before the occurrence of the event is demonstrated to be more probable than not. Nor is there necessarily a great gulf between proof in civil and criminal matters. In matters of proof the law looks for probability, not certainty. In some criminal cases, liberty may be involved; in some it may not. In some civil cases – as here – the issues may involve questions of character and reputation and the ability to pursue one’s chosen career that can approach, if not transcend in importance even questions of personal liberty. The gravity of the allegations and the related probability or improbability of their occurrence become in effect part and parcel of the circumstances which must be weighed in deciding whether, on balance, they are true.

Without deciding the matter, the Panel notes that it appears that this is the very sort of approach contemplated by Article 3.1 of the World Anti-Doping Code, which refers to a standard of proof “bearing in mind the seriousness of the allegation which is made” and which further states that “[t]his standard of proof in all cases is greater than a *mere* balance of probability ...” (Emphasis added)

From this perspective, and in view of the nature and gravity of the allegations at issue in these proceedings, there is no practical distinction between the standards of proof advocated by USADA and the Respondents. It makes little, if indeed any, difference whether a “beyond reasonable doubt” or “comfortable satisfaction” standard is applied to determine the claims against the Respondents. This will

become all the more manifest in due course, when the Panel renders its awards on the merits of USADA's claims. Either way, USADA bears the burden of proving, by strong evidence commensurate with the serious claims it makes, that the Respondents committed the doping offences in question.

H. The Hearing on the Merits (6 - 10 June 2005)

37. On 28 April 2005, Claimant filed a motion to postpone the hearing that was scheduled on 6 June 2005. USADA requested that the hearing be postponed until after the conclusion of the BALCO criminal trial so as to ensure, to the extent possible, that Victor Conte (who refused to testify in these proceedings prior to the completion of the BALCO trial) and IRS Agent Jeff Novitzky (whose testimony in these proceedings prior to the BALCO trial was "uncertain") would be available to testify before the Panel. Claimant's Motion was denied, as much for the fact that USADA had long been aware of the possibility that Messrs. Conte and Novitzky might not be available to testify in the arbitration as for the patent unfairness to Respondent that would be caused by any additional delay in the resolution of the charges brought against him.

38. As previously agreed and set out in the Panel's Orders of 1 and 9 November 2004, the hearing on the merits in this case took place in San Francisco during the 5-day period from 6-10 June 2005.

39. At the hearing, the Panel heard oral argument from both parties. It also heard the evidence of the following witnesses:

For USADA

- Dr Larry Bowers, USADA's Senior Manager Director, who testified regarding the evidence discovered during the BALCO investigation as well as regarding Mr. Montgomery's blood and urine testing;
- IRS Agent Jeff Novitzky, who gave evidence regarding the BALCO investigation and the documents discovered in the course of that investigation;

- Ms. Kelli White, a former elite American athlete who has admitted to doping with the assistance of BALCO, who testified regarding an alleged admission made to her by Mr. Montgomery;
- Dr Hans Geyer, an expert who testified with respect to Respondent's urine test results;
- Dr Richard Clark, an expert called to analyze Mr. Montgomery's urine test results submitted by USADA; and
- Dr Michael Sawka, an expert called by USADA to give evidence regarding Mr. Montgomery's blood test results.

For Respondent

- Dr. David Black, President and Laboratory Director of Aegis Sciences Corp. and Aegis Analytical Laboratories, who provided expert evidence regarding the analytical laboratory data (blood and urine tests) produced by USADA; and
- Dr. James Stray-Gundersen, an expert who testified regarding the blood testing results for Mr. Montgomery produced by USADA.

40. Although Mr. Montgomery's counsel cross-examined each of the witnesses produced by USADA, the Athlete called no fact witnesses of his own nor did he himself give evidence.

IV. THE CASE AGAINST MR. MONTGOMERY

I. Applicable IAAF Rules

41. As set out in USADA's Charging Letter and Statement of Claim, the charges brought against the Respondent concern alleged offences under IAAF Rules 55.2, 56.3, 56.4, and 60.1 (reproduced in full in paragraph 14 above). As noted, these charges are brought under the 2002 edition of the IAAF Rules (IAAF Official Handbook 2002-2003), which are applicable.

42. Notwithstanding the breadth of the charges brought against Mr. Montgomery – comprising the *presence, use* and *admission* of use of prohibited substances or techniques (Rules 55.2 and 60.1), *assisting* and *inciting* others to do so (Rules 56.3 and 60.1), and *trafficking* in prohibited substances (Rules 56.4 and 60.1) – it became increasingly apparent in the course of the proceedings, that the thrust of USADA's case concerns allegations of the use of prohibited substances and techniques (including alleged admissions of use and evidence of the presence of prohibited substances in the Athlete's body) as opposed to the "assisting or inciting" and "trafficking" charges. Ultimately, these charges were dropped by USADA.

J. USADA's 7 Types of Evidence

43. As presented by USADA at the hearing, the evidence of doping by Mr. Montgomery consisted of what Claimant referred to as 7 types of evidence:

- (1) Blood test results from a Mexican laboratory in February 2000 which allegedly show Mr. Montgomery's testosterone level doubling in the course of one day;
- (2) Documents extracted from the files seized from BALCO which, according to USADA, "individually or when linked together established Montgomery's doping";
- (3) Evidence of the suppression and rebound of endogenous steroids in Respondent's urine, as shown in a table depicting test results reported by IOC-accredited and BALCO Laboratories on 56 occasions between March 1999 and September 2004;
- (4) Alleged abnormal blood test results on 5 occasions between November 2000 and July 2001;
- (5) Respondent's alleged admission to Kelli White that he had used a prohibited substance known colloquially as the "Clear";
- (6) So-called admissions against interest, which implicated Mr. Montgomery, made by the President of BALCO, Victor Conte, in interviews with investigative authorities as well as the media; and

- (7) Reports in the San Francisco Chronicle supposedly based on secret grand jury testimony by Mr. Montgomery in which he admits to using various prohibited substances.

44. All of the foregoing evidence was challenged by the Respondent. This includes the reliability and veracity of statements regarding Mr. Montgomery contained both in statements that may have been made by Victor Conte and in documents found in his files, the propriety of the Panel considering newspaper reports allegedly derived from secret Grand Jury testimony, the credibility of Ms. White's testimony before the Panel in this arbitration and, significantly, the authenticity, reliability, interpretation and weight of test results conducted by non-IOC-accredited labs as well as the overall interpretation of the numerous blood and urine test results for Mr. Montgomery.

45. The Panel has wrestled with the question whether, in the circumstances, it should address in this Award each element of USADA's case against Mr. Montgomery, including each of what USADA calls its "7 types of evidence" of doping by the Athlete. On balance, the Panel has determined not to do so for the simple reason that it is unnecessary. This is because the Panel is unanimously of the view that Mr. Montgomery in fact admitted his use of prohibited substances to Ms. White, as discussed in more detail below, on which basis alone the Panel can and does find him guilty of a doping offence. The fact that the Panel does not consider it necessary in the circumstances to analyse and comment on the mass of other evidence against the Athlete, however, is not to be taken as an indication that it considers that such other evidence could not demonstrate that the Respondent is guilty of doping. Doping offences can be proved by a variety of means; and this is nowhere more true than in "non-analytical positive" cases such as the present.

K. Kelli White's Testimony

46. As mentioned, Ms. White has admitted to doping and has accepted a two-year sanction as a result. Having seen Ms. White and heard her testimony, including in response to questions put to her by counsel and the Panel, the members of the Panel do not doubt the veracity of her evidence. She answered all questions, including in relation to her own record of doping, in a forthright, honest and reasonable manner. She neither exaggerated nor sought to play down any aspect of her evidence. Clearly an intelligent woman, she impressed the

Panel with her candour as well as her dispassionate approach to the issues raised in her testimony and regarding which she was questioned by counsel and members of the Panel. In sum, the Panel finds Ms. White's testimony to be wholly credible.

47. According Ms. White's evidence, in March 2001, while at an international meet in Portugal (no exact date was provided by the witness) she and Mr. Montgomery had "a small discussion about whether or not the Clear made our calves tight." Mr. Montgomery asked Ms. White, "Does it make your calves tight?" Ms. White responded in the affirmative. Mr. Montgomery, still in her presence, then placed a telephone call to someone who may or may not have been Mr. Conte (Ms. White believes that it was Mr. Conte) to whom he relayed the information that "she said that it makes her calves tight too". According to Ms. White, there was not the slightest doubt as to the substance about which she and Mr. Montgomery were speaking and which they both acknowledged had the effect of making their calves tight: they were talking about the Clear.

48. It is essential to note that this evidence of what USADA claims constitutes a direct admission of Mr. Montgomery's guilt, is uncontroverted.

49. Counsel for Respondent may have questioned Ms. White's motives in offering her testimony concerning Mr. Montgomery's use of the Clear and, more generally, his relationship with BALCO. They may have sought (without success) to impugn her honesty and to draw attention to the witness' own history of involvement with BALCO and her efforts to conceal that involvement. However, the Panel has already declared its finding with respect to Ms. White's credibility as a witness in these proceedings and its view that she is telling the truth.

50. What counsel for Mr. Montgomery did not do was in any way undermine Ms. White's evidence regarding her conversation with Mr. Montgomery in March 2001. The evidence of that conversation, which the Panel considers to be clear and compelling, thus stands uncontroverted. It is also, as indicated above, sufficient in and of itself to find Respondent guilty of doping.

L. Mr. Montgomery's Decision Not to Testify

51. Of course, as noted by USADA's counsel during closing argument: "It would be a real different issue if Tim Montgomery took the stand and said '*no, no, when I said "it" I meant*

something else'." It might indeed have affected the Panel's appreciation of Ms. White's evidence had Respondent chosen to provide the Panel with a different explanation of their March 2001 conversation or had he denied that the conversation took place as described by the witness. The fact remains that he did not.

52. The Respondent's decision not to testify at his hearing did not come as a surprise. Indeed, the decision had been communicated to USADA and the Panel by Mr. Montgomery's counsel early in the proceedings. Nor is there any dispute as to Respondent's right to decide not to testify. It is common ground that Mr. Montgomery was fully within his rights to testify in his own defence, or not, as he saw fit. Where the parties differ, however, is with respect to the question (on which extensive pre-hearing submissions and authorities were filed and arguments were made during the hearing) whether the Panel has the authority to draw an adverse inference from Mr. Montgomery's decision not to testify in the arbitration; and, if it does have the power to do so, whether such an inference should be drawn in this case.

53. On 17 September 2005, the Panel advised the parties that, having considered their written and oral arguments and the legal authorities filed by them for and against the drawing of an adverse inference, and after deliberation, it found that "it does have the right and power to draw an adverse inference from Mr. Montgomery's refusal to testify. More particularly, it may draw adverse inferences in respect of allegations regarding which USADA has presented evidence that would normally call for a Response from the Respondent himself, and not merely from his experts or counsel." The Panel further informed the parties that it had not yet determined whether it would draw any such inferences and that its deliberations had been suspended so as to allow Respondent the opportunity to reconsider, in the circumstances, his decision not to testify.¹⁴

¹⁴ As explained in the Panel's 17 September letter, this somewhat unusual procedure was considered necessary and appropriate in the circumstances, so as to preserve the procedural harmony as between Mr. Montgomery's and Ms. Gaines' cases. As the Panel explained to the parties (and as USADA was well aware, in its capacity as the Claimant in the Gaines arbitration), because of the different manner in which events at her hearing unfolded, Ms. Gaines had had the opportunity to address the question whether, in the event that the Panel were to find that it may draw adverse inferences from her refusal to testify, she would wish to be so informed in order to be able to reconsider her decision. Ms. Gaines' answer was "No". The same opportunity for Mr. Montgomery to address this question had not arisen during his hearing the month before.

54. It is noted that in the case of *USADA v. Michelle Collins* the Arbitral Tribunal found that it "may draw certain adverse inferences" from the Respondent's refusal to testify, though "there is no rule obligating a Tribunal to draw an adverse inference." Indeed, the Tribunal went on to hold that "no adverse inference is necessary" given that the weight of the evidence "is already adverse to Collins so no further adverse inference need be drawn".

55. The situation is similar in the present case. Mr. Montgomery has been provided every conceivable opportunity to provide an exculpatory explanation of his own statements evidencing his guilt. He has had ample opportunity to deny ever making such statements. But because he has not offered any evidence of his own concerning his admission to Ms. White of his use of the Clear, the Panel can only rely on the testimony of Ms. White. That testimony is more than merely adverse to Mr. Montgomery; it is fatal to his case. In the circumstances, faced with uncontroverted evidence of such a direct and compelling nature, there is simply no need for any additional inference to be drawn from the Respondent's refusal to testify. The evidence alone is sufficient to convict.

V. DECISION

M. The Doping Offence

56. In its 4 March 2005 Decision on Evidentiary and Procedural Issues, the Panel observed that "it makes little, if indeed any difference, whether a 'beyond reasonable doubt' or 'comfortable satisfaction' standard is applied to determine the claims against the [Respondent] ... Either way, USADA bears the burden of proving, by strong evidence commensurate with the serious claims it makes that the [Respondent] committed the doping offences in question."

57. USADA has met this standard. The Panel has no doubt in this case, and is more than comfortably satisfied, that Mr. Montgomery committed the doping offence in question. It has been presented with strong, indeed uncontroverted, evidence of doping by Mr. Montgomery, in the form of an admission contained in his statements made to Ms. White and to others while in her presence. On this basis, the Tribunal finds Respondent guilty of a doping offence. In particular, the Panel finds Mr. Montgomery guilty of the offence of admitting having used a prohibited substance under IAAF Rules 55.2(iii) and 60.1(iii).

N. The Sanction

58. By way of sanction, USADA commenced this case by informing Mr. Montgomery that it intended to request, and indeed it requested from the Panel, "a lifetime period of ineligibility beginning on the date you accept this sanction or the date of the hearing panel's decision."¹⁵ It subsequently amended this request (including as a consequence of its withdrawal of the "trafficking" allegations against Respondent) and, at the close of the hearing, requested a four-year period of ineligibility.

59. USADA request is based on IAAF Rule 60.2 (a) (i), which provides that for a first offence under Rule 60.1(iii) (which includes the offence of admitting having used a prohibited substance) an athlete shall be ineligible "for a minimum of two years from the date of the hearing at which it is decided that a Doping Offence has taken place."

60. In the circumstances, the Panel finds that Mr. Montgomery's admission of his use of prohibited substances merits a period of ineligibility under IAAF Rules of two years.

61. This period of ineligibility shall commence to run as of 6 June 2005, being the first day of Mr. Montgomery's hearing. The Panel is of the view that this date of commencement of the sanction is fair and appropriate in the particular circumstances of this case in view of the numerous delays in the hearing process unattributable to the Athlete, including as a result of the agreement of all parties that Mr. Montgomery's and Ms. Gaines' cases should be run in tandem. Although this agreement entailed significant efficiencies overall, and doubtless permitted the two cases to be heard and decided (by the same Panel) more quickly than if they had been conducted sequentially, it inevitably meant that there would be some additional delay before either case could be heard.¹⁶

62. In addition to the two-year sanction already discussed, the Panel orders the retroactive cancellation of all of Mr. Montgomery's results, rankings, awards and winnings as of 31 March 2001 (as noted above, Ms. White did testify as to the exact date during the month of March, 2001 on which Mr. Montgomery admitted his use of the Clear, and the Panel thus

¹⁵ Quoted from USADA's Charging Letter : see above.

¹⁶ The agreement to maintain what the Panel has had occasion to refer to as the "procedural harmony" between the two cases also meant that the decision in the first of the two cases to be heard – which, as agreed between the parties would be Mr. Montgomery's case – would be issued by the Panel at the same time as its decision in the second case.

considers it reasonable that the last day of the month in question be selected for this purpose). In this regard, IAAF Rule 60.5 provides: "Where an athlete has been declared ineligible he shall not be entitled to any award or addition to his trust fund to which he would have been entitled by virtue of his appearance and/or performance at the athletics meeting at which the doping offence took place, or at any subsequent meetings."

VI. CONCLUSION

63. In its introduction to the present Award, the Panel described the relative novelty of this case, in which USADA sought to prove a doping offence in the absence of any "adverse analytical finding". It must also be noted that this case can be distinguished from those of other elite track athletes involved with BALCO, such as Ms. White, Alvin Harrison and Regina Jacobs, who admitted their guilt to USADA in the context of anti-doping proceedings.

64. The Panel would add, in conclusion, that there is no reason to believe that the world of sport has seen the last of this sort of "no adverse analytical finding" case. It must constantly be borne in mind, as noted above, that doping offences can be proved by a variety of means. In this regard, the Panel concurs with the observation expressed in the Comitato Olimpico Nazionale Italiano ("CONI") matter, that "in anti-doping proceedings other than those deriving from positive testing, sports authorities do not have an easy task in discharging the burden of proving that an anti-doping rule violation has occurred, as no presumption applies." However, the Panel also concurs wholeheartedly with the exhortation of the CONI Panel, which wrote as follows in the concluding passage of its Award, a declaration that this Panel adopts as its own:

In any event, the undeniable circumstance that the conviction for doping offences is more difficult when the evidence is other than positive testing must not prevent the sports authorities from prosecuting such offences, as already remarked, with the outmost earnestness and eagerness, using any available method of investigation. In the end, it will be up to the adjudicating body having jurisdiction over the matter – which, according to Article 8 of the WADC, must always be a "fair and impartial hearing body" – to determine case by case whether the standard of proof of Article 3.1 of the WADC has been met and the burden of proof has been discharged, or not, by the prosecuting sports authority.

VII. COSTS

65. The issue of costs is dealt with in paragraph 12 of the 8 September 2004 CAS Order of Procedure as follows, in terms that neither party has asked the Panel to disturb:

12.1 In accordance with art. 64 of the Code and with art. 9 b (iv) of the USADA Protocol, the costs of this arbitration will be borne by USADA.

12.2 Each party is responsible for the fees and costs of its lawyer and such costs as arise from the appearance of witnesses whose hearing has been requested.

VIII. PUBLICATION OF THE AWARD

66. In accordance with clause 13 of the Order of Procedure dated 8 September 2004, the award and a press release setting forth the outcome of the proceedings shall be made public by the CAS.

ON THESE GROUNDS

The Panel unanimously finds and orders as follows:

1. Respondent is guilty of the offence of admitting having used a prohibited substance under IAAF Rules 55.2(iii) and 60.1(iii);
2. The following sanctions shall be imposed on Respondent:
 - a. A period of ineligibility under IAAF Rules for two years commencing as of 6 June 2005, including his ineligibility from participating in U.S. Olympic, Pan American or Paralympic Games, trials or qualifying events, being a member of any U.S. Olympic, Pan American or Paralympic Games team and having access to the training facilities of the United States Olympic Committee ("USOC") Training Centers or other programs and activities of the USOC including, but not limited to, grants, awards, or employment pursuant to the USOC Anti-Doping Policies;
 - b. The retroactive cancellation of all awards or additions to Respondent's trust fund to which he would have been entitled by virtue of his appearance and/or performance at any athletics meeting occurring between 31 March 2001 and the date of this Award;
3. The costs of the arbitration, to be determined and notified to the parties by the Secretary General of the CAS in accordance with article R 64.4 of the CAS Code, shall be borne by USADA;
4. Each party shall bear all of its own costs, including the fees and expenses of its lawyers and witnesses;
5. This Award deals definitively with all charges brought against Respondent by Claimant in this arbitration. All charges not expressly dealt with herein are dismissed.

Lausanne, 13 December 2005

THE COURT OF ARBITRATION FOR SPORT

L. Yves **Fortier**, CC, QC
President of the Panel

Christopher Campbell
Arbitrator

Peter Leaver QC
Arbitrator

Stephen Drymer
Ad hoc Clerk

**TAS 2006/A/1119 Union Cycliste Internationale (UCI) c/ Iñigo Landa Intxaurreaga
& Real Federación Española de Ciclismo (RFEC)**

SENTENCE ARBITRALE

rendue par le

TRIBUNAL ARBITRAL DU SPORT

siégeant dans la composition suivante :

Président : Maître Jan **Paulsson**, avocat à Paris, France
Arbitres : Maître Olivier **Carrard**, avocat à Genève, Suisse
Maître José Juan **Pintó**, avocat à Barcelone, Espagne
Greffier ad hoc : Maître Shaparak Saleh, avocat à Paris, France

dans l'arbitrage entre

L'Union Cycliste Internationale (UCI), Aigle, Suisse
représentée par Maître Philippe Verbiest, avocat à Leuven, Belgique

contre

Monsieur Iñigo Landa Intxaurreaga, Espagne
représenté par Maître Carmen Coello de Portugal Silva et Maître Immaculada Herranz
Perlado, avocats à Madrid, Espagne

et

Real Federación Española de Ciclismo (RFEC), Madrid, Espagne
représentée par Maître José Antonio del Valle Herán, avocat à Madrid, Espagne

* * * * *

I. LES FAITS

1. L'Union Cycliste Internationale (l'**UCI**) est l'association des fédérations nationales du cyclisme. Elle a pour but la direction, le développement, la réglementation, le contrôle et la discipline du cyclisme. Le système de contrôle antidopage mis en place par l'UCI comprend notamment le Règlement du Contrôle Antidopage (le **RCAD**), la liste des classes de substances dopantes et des méthodes de dopage, la liste des épreuves internationales à contrôler ainsi que la liste des laboratoires accrédités.
2. La Real Federación Española de Ciclismo (la **RFEC**), fédération nationale espagnole chargée du cyclisme et dotée d'un règlement disciplinaire au niveau national, est membre de l'UCI.
3. Monsieur Iñigo Landaluce Intxaurreaga (**Monsieur Landaluce**) est un coureur cycliste espagnol de la catégorie élite, titulaire d'une licence délivrée par la RFEC. Membre à l'époque des faits de l'équipe cycliste Euskaltel, il a participé à la course cycliste internationale sur route « Critérium du Dauphiné Libéré » qui s'est déroulée du 5 au 12 juin 2005.
4. A l'issue de l'étape du 11 juin 2005, Monsieur Landaluce a fait l'objet d'un contrôle antidopage. Au cours du prélèvement de l'échantillon, Monsieur Landaluce a indiqué avoir pris de la triamcinolone en infiltration le 21 mars 2005.
5. L'échantillon A prélevé sur Monsieur Landaluce a été analysé par le laboratoire français de dépistage du dopage de Châtenay-Malabry (le **LNDD**), laboratoire accrédité par l'Agence Mondiale Antidopage (l'**AMA**). Son rapport d'analyse du 17 juillet 2005 a révélé que « *l'analyse par spectrométrie de masse de rapports isotopiques indique une prise de Testostérone ou de l'un de ses précurseurs* ». Le 28 juillet 2005, le rapport d'analyse relatif à l'échantillon A a été transmis à la RFEC, afin que soit mise en œuvre une procédure disciplinaire, conformément à l'article 187 du RCAD.
6. Le 11 août 2005, Monsieur Landaluce a, par l'intermédiaire de la RFEC, sollicité une contre-analyse. Il a ensuite demandé que la contre-analyse qui était initialement prévue les 14 et 21 septembre 2005, soit reportée au 19 octobre 2005. Le 21 octobre 2005, la contre-analyse a confirmé la présence de testostérone de nature exogène dans l'échantillon B.

7. Par télécopie du 27 octobre 2005, l'UCI a transmis les résultats d'analyse de l'échantillon B à la RFEC et lui a demandé de poursuivre la procédure disciplinaire.
8. Le 5 mai 2006, la RFEC, par l'intermédiaire du Comité Nacional de Competición y Disciplina Deportiva (Comité national de compétition et discipline sportive / *CNCDD*), a jugé que les conditions dans lesquelles l'analyse avait été effectuée révélaient l'existence « *d'un processus incomplet pour ne pas être conforme à toutes les exigences légales applicables et, ainsi, ne pouvant pas garantir totalement le résultat* ». Le CNCDD a dès lors estimé que « *la maxime juridique « in dubio pro reo » [était] pleinement applicable au cas présent* », a classé l'affaire et acquitté Monsieur Landaluce.
9. Monsieur Landaluce n'a participé à aucune course cycliste entre le 24 juillet 2005 et le 15 mai 2006.

II. LA PROCÉDURE DEVANT LE TRIBUNAL ARBITRAL DU SPORT

A. LES DEMANDES ET REPONSES DES PARTIES

10. Par déclaration du 26 juin 2006, l'UCI a saisi le Tribunal Arbitral du Sport (le *TAS*).

Le 17 juillet 2006, l'UCI a déposé son mémoire d'appel demandant au TAS :

- « 1) *de réformer la décision du Comité national de compétition et discipline sportive de la RFEC ;*
- 2) *de condamner M. Landaluce Intxaurreaga à une suspension de 2 ans, conformément au RAD ;*
- 3) *de prononcer la disqualification de M. Landaluce Intxaurreaga des courses cyclistes auxquelles il a participé à partir du 11 juin 2005 et notamment de la course cycliste du « Critérium du Dauphiné Libéré 2005 », jusqu'au 29 juillet 2005 au moins ;*
- 4) *de condamner M. Landaluce Intxaurreaga et la Real Federacion Española de Ciclismo à payer à l'UCI un montant de CHF 1'000.- à titre de frais de gestion des résultats (art. 245.2 RAD) ;*
- 5) *de condamner M. Landaluce Intxaurreaga et la Real Federacion Española de Ciclismo, solidairement à rembourser à l'UCI l'émolument de CHF 500.- et à tous les autres frais, y compris une contribution aux frais de l'UCI ».*

11. Monsieur Landaluce a déposé sa réponse le 8 septembre 2006, aux termes de laquelle il demande au TAS de :

- se déclarer incompétent ;
- condamner l'UCI au paiement des frais générés par la procédure.

A titre subsidiaire, Monsieur Landaluce sollicite du TAS qu'il :

- rejette l'appel interjeté par l'UCI ;
- ne prononce aucune sanction à son encontre ;
- condamne l'UCI au paiement des frais.

A titre encore plus subsidiaire, Monsieur Landaluce fait valoir que toute sanction prononcée à son encontre devra être réduite de 9 mois et demi, période pendant laquelle il s'est abstenu de courir.

12. La RFEC a déposé sa réponse le 14 septembre 2006, faisant valoir en substance que :

- la décision de la CNCDD doit être confirmée ;
- l'UCI doit être déboutée de son appel ;
- l'UCI doit être condamnée aux dépens, en application de l'article 282 du RCAD.

B. L'AUDIENCE

13. L'audience a eu lieu le 11 octobre 2006, au siège du TAS à Lausanne (l'*Audience*), en présence des trois membres du Tribunal Arbitral (la *Formation*) et des avocats des parties.

14. La Formation a entendu les témoins et experts suivants :

- Pour l'UCI :
 - Docteur Martial Saugy, directeur du laboratoire suisse d'analyse du dopage, (*Dr Saugy*) ;
 - Professeur Jacques de Ceaurriz, directeur du LNDD (*Prof. de Ceaurriz*).
- Pour Monsieur Landaluce :

- Docteur Douwe de Boer, chercheur au Laboratoire de chimie clinique à l'Hôpital Universitaire de Maastricht et ancien directeur du Département dopage du Laboratoire d'analyses et de dopage de Lisbonne (*Dr de Boer*);
- Docteur Detlef Thieme, spécialiste en toxicologie médico-légale au sein du département de toxicologie médico-légale de l'Institut de médecine légale de Munich (*Dr Thieme*);
- Monsieur Ignacio Colomer Hernández, Professeur de droit à l'Université Carlos III de Madrid (*Prof. Colomer*), entendu par vidéoconférence.

C. S'AGISSANT DE LA PRODUCTION DES PIÈCES 43 ET 43 BIS PAR MONSIEUR LANDALUCE ET DE L'AUDITION DE MONSIEUR MIGUEL MADARIAGA

15. Le 22 septembre 2006, soit 14 jours après avoir déposé sa réponse, Monsieur Landaluce a déposé les pièces complémentaires 43 et 43 bis.
16. L'UCI s'étant opposée à la production desdites pièces le 25 septembre 2006, Monsieur Landaluce a indiqué, par télécopie du 2 octobre 2006, qu'il souhaitait faire entendre comme témoin Monsieur Miguel Madariaga (*Monsieur Madariaga*).
17. Par télécopie du 4 octobre 2006, l'UCI s'est opposée à l'audition de Monsieur Madariaga, la réponse de Monsieur Landaluce ne mentionnant aucunement l'audition de ce témoin.
18. Aux termes de l'article R56 du Code de l'arbitrage en matière de sport :

« Sauf accord contraire des parties ou décision contraire du Président de la formation commandée par des circonstances exceptionnelles, les parties ne sont pas admises à compléter leur argumentation, ni à produire de nouvelles pièces, ni à formuler de nouvelles offres de preuves après la soumission de la motivation d'appel et de la réponse ».
19. Lors de l'Audience, l'UCI a indiqué qu'elle ne donnerait son accord à l'audition de Monsieur Madariaga que si elle pouvait verser aux débats une nouvelle pièce reçue la veille. Le Président de la Formation a demandé que la pièce soit transmise à la RFEC et à Monsieur Landaluce afin que ceux-ci en prennent connaissance. Au vu de la pièce, Monsieur Landaluce a renoncé à faire entendre Monsieur Madariaga.
20. En l'absence de « *circonstances exceptionnelles* » au sens de l'article R56 du Code de l'arbitrage en matière de sport, le Président de la Formation a décidé de ne pas admettre

les nouvelles pièces et de ne pas procéder à l'audition du témoin.

III. S'AGISSANT DE LA COMPÉTENCE DU TAS

21. L'organisation de compétitions professionnelles internationales suppose une répartition claire des compétences entre les autorités nationales et internationales, d'une part, entre le secteur public et le secteur privé, d'autre part.
22. Il est des domaines, tel que le trafic de stupéfiants, pour lesquels chaque État exige, au nom de la souveraineté nationale, de contrôler lui-même tout comportement répréhensible sur son territoire, et refuse de laisser à des associations privées le soin de s'en charger, fût-ce par le biais d'une délégation de service public.
23. En revanche, la discipline en matière sportive, qui se traduit par la disqualification de l'athlète ou par l'interdiction qui lui est faite de participer à de futures compétitions, répond essentiellement à la volonté d'éliminer toute concurrence déloyale. C'est pourquoi elle est légitimement confiée à des autorités sportives.
24. Cela est d'autant plus vrai s'agissant de l'exercice d'un sport au niveau international, qui ne saurait obéir à une multiplicité de règles nationales potentiellement contradictoires.
25. Confier aux seules lois nationales le soin de régir les conditions dans lesquelles doivent se dérouler les compétitions internationales aboutirait à un système incohérent et inégalitaire, avec de surcroît le risque que les autorités nationales se livrent tôt ou tard à une course à la législation la moins répressive.
26. Pour pallier de tels inconvénients il suffit de s'assurer que la même discipline sportive, lorsqu'elle se déroule dans un cadre international, est soumise aux mêmes règles pour tous les concurrents.
27. Or, Monsieur Landaluce tente de faire accroire que la situation juridique en Espagne dicterait une solution différente. Il fait en effet valoir que le TAS ne serait pas compétent pour connaître de l'appel de l'UCI contre une décision du CNCDD et indique que le Comité Espagnol de Discipline Sportive du Conseil Supérieur des Sports Espagnols (le *CEDS*) serait seul compétent.
28. Au soutien de sa prétention, Monsieur Landaluce invoque un certain nombre de textes qui sont repris ci-après :

- L'article 6 des Statuts de l'UCI :
 - « 1. Les fédérations s'engagent, du fait de leur affiliation, à se conformer aux statuts et règlements de l'UCI ainsi qu'à toute décision prise conformément à ceux-ci. De même, elles s'engagent à faire respecter les statuts, règlements et décisions de l'UCI par toute personne concernée.
 - 2. Les règlements de l'UCI doivent être repris dans les règlements correspondants des fédérations.
 - 3. Les statuts et règlements des fédérations ne peuvent aller à l'encontre de ceux de l'UCI. En cas de divergence, seuls les statuts et les règlements de l'UCI seront appliqués. Les statuts et les règlements des fédérations doivent contenir la clause expresse qu'en cas de conflit avec les statuts ou règlements de l'UCI, seuls ces derniers seront appliqués.
 - 4. Les alinéas ci-dessus sont appliqués dans le respect des dispositions de droit impératif en vigueur dans le pays de la fédération concernée ».
- L'article 35 du Décret Royal 1835/1991 du 20 décembre 1991 relatif aux fédérations sportives espagnoles :
 - « Les questions suivantes ne pourront pas faire l'objet d'une conciliation ou d'un arbitrage : [...] »
 - b) Celles qui seront en relation avec le contrôle des substances et des méthodes prohibées dans le sport et la sécurité de la pratique sportive [...] ».
- L'article 90.2 des Statuts de la RFEC dans sa rédaction issue de la décision du 11 octobre 2004 :
 - « Les questions suivantes ne pourront pas faire l'objet d'une conciliation : [...] »
 - b) Celles qui seront en relation avec le contrôle des substances et des méthodes prohibées dans le sport et la sécurité de la pratique sportive [...] ».
- L'article 24 de la Constitution espagnole du 28 décembre 1978 :
 - « 1. Toute personne a le droit d'obtenir la protection effective des juges et des tribunaux pour exercer ses droits et ses intérêts légitimes sans, qu'en aucun cas, cette protection puisse lui être refusée.
 - 2. De même, toute personne a le droit d'aller devant le juge ordinaire déterminé préalablement par la loi, de se défendre et de se faire assister par un avocat, d'être informée de l'accusation portée contre elle, d'avoir un procès public sans délai indu et avec toutes les garanties, d'utiliser les preuves nécessaires à sa défense, de ne pas faire de déclaration contre elle-même, de ne pas s'avouer coupable et d'être présumée innocente ».
- L'article 91.1 des Statuts de la RFEC dans sa rédaction issue de la décision du 11 octobre 2004 :
 - « La déclaration de volonté irréfutable des parties au litige de se soumettre à la procédure de conciliation sera exigée. Pour cela, une convention

d'arbitrage sera souscrite par écrit et devra exprimer la renonciation à la voie judiciaire, l'obligation de se soumettre à la décision d'un ou de plusieurs arbitres et aux règles de procédure ».

- L'article 84.1 de la Loi 10/1990 du 15 octobre 1990 relative au Sport :
« Le Comité Espagnol de Discipline Sportive est l'organe national affecté organiquement au Conseil Supérieur des Sports qui, agissant indépendamment de celui-ci, tranche en dernier recours, par voie administrative, les litiges portant sur les questions disciplinaires sportives qui relèvent de son ressort ».
- L'article 10 des Statuts de la RFEC :
« 1. Sous la coordination et la tutelle du Conseil Supérieur des Sports, la RFEC exercera les fonctions publiques de nature administrative suivantes : [...]
f) Exercer l'autorité disciplinaire sportive, selon les termes établis par la Loi sur le Sport, ses dispositions particulières de développement et ses statuts et règlements ».
- L'article 59 du Décret Royal 1591/1992 du 23 décembre 1993 :
« Les compétences du Comité Espagnol de Discipline Sportive s'étendent :
a. A la connaissance et décision, par voie de recours, des prétentions formulées en ce qui concerne les actes des organes sportifs titulaires de l'autorité disciplinaire qui épuisent la voie sportive, selon la répartition des compétences établie par la Loi sur le Sport et par le présent décret royal ».

29. Monsieur Landaluce prétend en substance :

- que le recours à l'arbitrage serait interdit en matière de dopage en Espagne ;
- que la Constitution espagnole érigerait un droit inaliénable d'accès à la justice et aux tribunaux. Monsieur Landaluce indique qu'il ne saurait être privé de ce droit constitutionnel d'accès à la justice en n'étant autorisé à porter son appel que devant le TAS ;
- ne jamais avoir manifesté sa volonté de se soumettre à l'arbitrage du TAS et n'avoir signé aucune convention d'arbitrage. Monsieur Landaluce estime que le simple fait de disposer d'une licence de la RFEC ne saurait valoir acceptation de la compétence du TAS ;
- que la RFEC serait une autorité publique exerçant des fonctions publiques disciplinaires sous la tutelle d'un organisme public espagnol. Monsieur Landaluce estime, en conséquence, que les décisions de la RFEC devraient

faire l'objet d'un recours administratif devant le CEDS puis, le cas échéant, devant le juge administratif espagnol ;

- qu'en vertu de l'article 6 alinéa 4 des Statuts de l'UCI, la RFEC doit respecter la législation espagnole lorsqu'elle applique les normes de l'UCI ;
- que les articles 242 et 280 à 285 du RCAD, qui établissent la compétence exclusive du TAS pour connaître de l'appel dirigé contre les décisions des fédérations nationales seraient inapplicables conformément à l'article 6.4 des Statuts de l'UCI, dès lors que cette compétence exclusive violerait le droit espagnol ;
- que l'arrêt du Tribunal central de contentieux administratif n° 7 de Madrid du 8 juin 2006 rendu dans une affaire Santiago Perez Fernandez reconnaît la possibilité de former un recours hiérarchique contre les décisions du CNCDD devant le CEDS puis, le cas échéant, devant le juge administratif espagnol.

30. L'UCI estime, pour sa part, que le TAS est compétent et que le RCAD est applicable, en se fondant sur les textes suivants :

- L'article 1.1.001 du Règlement du sport cycliste de l'UCI :
« La licence est une pièce d'identité qui confirme l'engagement de son titulaire à respecter les statuts et règlements et qui l'autorise à participer aux événements cyclistes ».
- L'article 1.1.004 du Règlement du sport cycliste de l'UCI :
« Toute personne demandant une licence s'engage de ce fait à respecter les statuts et les règlements de l'UCI, des confédérations continentales de l'UCI et des fédérations membres de l'UCI et à participer aux manifestations cyclistes d'une manière sportive et loyale. Elle s'engage notamment à respecter les obligations visées à l'article 1.1.023.
Dès la demande de licence et pour autant que la licence est délivrée, le demandeur est responsable des infractions aux règlements qu'il commet et soumis à la juridiction des instances disciplinaires.
Tout licencié reste soumis à la juridiction des instances disciplinaires compétentes pour les faits commis alors qu'il était demandeur ou titulaire d'une licence, même si la procédure est engagée ou se poursuit après le moment où l'intéressé n'a plus de licence ».
- L'article 1.1.023 du Règlement du sport cycliste de l'UCI :
« [...] 2. Je m'engage à respecter les statuts et règlements de l'Union Cycliste Internationale, de ses confédérations continentales et de ses fédérations nationales.

Je déclare avoir lu ou avoir eu la possibilité de prendre connaissance de ces statuts et règlements.

Je participerai aux compétitions ou manifestations cyclistes d'une manière sportive et loyale.

Je me soumettrai aux sanctions prononcées à mon égard et porterai les appels et litiges devant les instances prévues aux règlements. J'accepte le Tribunal Arbitral du Sport (TAS) comme seule instance d'appel compétente dans les cas et suivant les modalités prévues par les règlements.

J'accepte que le TAS se prononce en dernière instance et que ses décisions seront définitives et sans appel. Sous ces réserves, je soumettrai tout litige éventuel avec l'UCI exclusivement aux tribunaux du siège de l'UCI.

3. J'accepte de me soumettre à et être lié par le règlement antidopage de l'UCI, les clauses du Code Mondial Antidopage et ses Standards internationaux auxquels le règlement antidopage de l'UCI fait référence ainsi que les règlements antidopage des autres instances compétentes suivant les règlements de l'UCI et le Code Mondial Antidopage, pour autant qu'ils soient conformes à ce Code. [...] ».

- L'article 5 des dispositions préliminaires du Règlement du sport cycliste de l'UCI :

« La participation à une épreuve cycliste, à quelque titre que ce soit, vaut acceptation de toutes les dispositions qui y trouvent application ».

31. L'UCI indique, en substance, qu'il ressort de la lecture combinée des articles précités que :

- les licenciés des fédérations membres de l'UCI sont soumis au RCAD ;
- toute personne qui demande une licence s'engage à respecter le RCAD et s'engage notamment à se soumettre aux contrôles antidopage et à la compétence du TAS en dernier ressort.

32. Au soutien de sa prétention, l'UCI rappelle que la décision du CNCDD du 5 mai 2006 dont il est fait appel a été rendue en application du RCAD.

33. En outre, lors de l'Audience, l'UCI a fait valoir que :

- le TAS devrait apprécier la compétence et l'arbitrabilité des litiges selon la loi fédérale suisse sur le droit international privé (**LDIP**) aux termes de laquelle (i) le dopage est arbitral, et (ii) les clauses d'arbitrage par référence sont valables (article 178 de la LDIP) ;
- selon la jurisprudence du TAS, l'existence d'une loi nationale n'empêcherait pas l'application de la réglementation de la fédération internationale (TAS 2005/A/872, sentence du 30 janvier 2006, UCI / Federico Muñoz Fernandez et Federación Colombiana De Ciclismo) ;

- la législation espagnole invoquée par Monsieur Landaluce n'aurait vocation à s'appliquer qu'en Espagne, comme le révéleraient :
 - (i) l'article 1.3° du Décret Royal 1835/1991 du 20 décembre 1991 sur les fédérations sportives espagnoles, aux termes duquel le champ de compétence des fédérations sportives espagnoles se limiterait au territoire national ;
 - (ii) le préambule de la Loi 10/1990 du 15 octobre 1990 sur le sport, selon lequel l'objectif de la loi serait de définir le cadre juridique applicable à la pratique sportive au sein du territoire espagnol.
 - l'article 7 du Décret Royal 255/1996 du 16 février 1996, aux termes duquel les sanctions imposées en application des règles antidopage d'une quelconque fédération, que celle-ci soit nationale, internationale ou autonome, produisent leurs effets sur l'ensemble du territoire espagnol, révélerait nécessairement que des sanctions peuvent être imposées par d'autres fédérations que la RFEC et produire leurs effets en Espagne ;
 - n'admettre comme seule voie de recours contre les décisions du CNCDD l'appel devant le CESD équivaldrait à priver l'UCI de tout droit de regard sur les décisions émanant de la fédération nationale, dans la mesure où l'UCI ne serait pas habilitée à former appel devant la juridiction espagnole.
34. Dans ses écritures et lors de l'Audience, la RFEC a indiqué se rallier à l'interprétation faite par l'UCI, s'agissant de la compétence du TAS. La RFEC a par ailleurs indiqué qu'elle était un établissement privé « à deux casquettes » exerçant, selon les cas, des missions d'ordre public ou d'ordre privé. La RFEC a précisé que, dans le cadre de compétitions internationales, comme au cas particulier, elle exerçait une mission d'ordre privé.
35. Lors de l'Audience, le Prof. Colomer a fait les observations suivantes :
- l'octroi d'une licence par la RFEC serait constitutif d'un acte administratif ;
 - le pouvoir de sanction de la RFEC interviendrait dans le cadre de l'exercice d'une fonction publique ;

- le TAS ne serait pas compétent car la sanction imposée par le CNCDD serait un acte administratif ;
 - l'interdiction du recours à l'arbitrage en matière de dopage serait impérative pour les parties, qui ne sauraient y déroger ;
 - la Constitution espagnole prévaudrait sur tout traité international ;
 - tout décret contraire à la Constitution espagnole serait anticonstitutionnel ;
 - l'article 7 du Décret Royal 255/1996 du 16 février 1996 serait anti-constitutionnel.
36. Consciente du fait que le présent litige illustre la tension qui peut exister entre la loi nationale d'un athlète et le règlement de la fédération internationale auquel celui-ci est soumis, la Formation rappelle que l'UCI est l'association des fédérations nationales du cyclisme qui a pour but la direction, le développement, la réglementation, le contrôle et la discipline du cyclisme international, notamment en matière de dopage. Monsieur Landaluce est, quant à lui, un cycliste professionnel de la catégorie élite, titulaire d'une licence aux termes de laquelle il a accepté de se soumettre aux règles de l'UCI.
37. Certes, la demande de licence de Monsieur Landaluce révèle que le modèle de demande de licence utilisé par la RFEC n'est pas conforme au règlement de l'UCI. Toutefois, les articles 2 et 3 de la demande de licence signée par Monsieur Landaluce indiquent que son détenteur s'engage à respecter la législation espagnole en vigueur, les Statuts de l'UCI ainsi que de ses confédérations, et qu'il accepte de se soumettre aux contrôles antidopage dans les conditions prévues à l'article 1.1.023 du Règlement du sport cycliste de l'UCI ainsi qu'à la compétence du TAS qui y est explicitement prévue. La RFEC n'a d'ailleurs pas manqué d'indiquer, lors de l'Audience, que le fait de lui demander une licence valait acceptation de la compétence du TAS, même en l'absence d'une clause expresse à cet effet. La Formation considère qu'en demandant sa licence à la RFEC, Monsieur Landaluce s'est soumis à la compétence du TAS, ainsi qu'aux règlements de l'UCI.
38. D'aucuns pourraient penser qu'il aurait été préférable que la demande de licence mentionne expressément la soumission des parties à l'arbitrage du TAS. A cet égard, la Formation souligne que les coureurs d'élite ne sont pas des profanes, contrairement aux consommateurs, mais des sportifs avertis qui ont sans le moindre doute connaissance de

l'existence du TAS et de sa compétence pour connaître des affaires de dopage. Dès lors, la compétence du TAS telle que prévue à l'article 1.1.023 du Règlement du sport cycliste ne saurait en aucun cas constituer une surprise pour la personne qui formule une demande de licence. Exiger qu'en sus des éléments rappelés au paragraphe 37 ci-dessus, la demande de licence mentionne expressément la compétence du TAS pour que celui-ci soit compétent, serait un excès de formalisme et une exigence non requise par la jurisprudence du Tribunal fédéral suisse.

39. L'argumentation de Monsieur Landaluce tendant à faire valoir que l'article 6 alinéa 4 des Statuts de l'UCI enjoindrait à la RFEC d'appliquer la loi espagnole et d'interdire le recours à l'arbitrage du TAS n'emporte guère la conviction de la Formation. En effet, l'article 6 des Statuts de l'UCI s'adresse aux seules fédérations nationales. Les trois premiers alinéas de cet article mettent à la charge de ces fédérations une obligation d'harmoniser leurs règles en reprenant les règlements de l'UCI. Le quatrième alinéa de l'article 6 des Statuts de l'UCI, indique simplement que cette harmonisation doit tenir compte d'éventuelles dispositions impératives applicables dans le pays de la fédération concernée. En conséquence, l'article 6 alinéa 4 des Statuts de l'UCI renvoie expressément aux trois paragraphes qui le précèdent et ne saurait être détaché de son contexte. L'article 6 des Statuts de l'UCI ne s'adresse donc qu'aux fédérations nationales, en vue de leur indiquer la démarche à suivre afin d'harmoniser leur droit, et ne saurait nullement être invoqué par un cycliste afin de se soustraire à l'application des règlements de l'UCI et, ce faisant, à la compétence du TAS.
40. Au demeurant, aucune disposition de la loi espagnole n'exclut le recours à l'arbitrage. Ce n'est certainement pas le cas de l'article 24 de la Constitution espagnole. Il suffit pour s'en convaincre de comparer la portée de ce texte à celle de l'article 6 alinéa 1 de la Convention européenne des droits de l'homme (la **CEDH**) qui prévoit que « *toute personne a droit à ce que sa cause soit entendue [...] par un tribunal indépendant et impartial, établi par la loi* ». Or, la Commission européenne des droits de l'homme a jugé dans une affaire X. / RFA du 5 mars 1962, que l'insertion d'une clause compromissoire dans un contrat valait renonciation au bénéfice de la CEDH et qu'aucune stipulation de la CEDH n'interdisait une telle renonciation. Sauf preuve du contraire, l'article 24 de la Constitution espagnole, tout comme l'article 6 alinéa 1 de la CEDH, n'interdit nullement le recours à l'arbitrage. En effet, la compétence du TAS ne

prive pas Monsieur Landaluce de son droit d'accès à la justice tel que prévu par la Constitution espagnole, dès lors qu'en matière internationale, le recours à l'arbitrage est une modalité d'exercice de ce droit.

41. La Formation considère en définitive, que les dispositions espagnoles invoquées par Monsieur Landaluce n'ont vocation à s'appliquer que dans le cadre de compétitions cyclistes nationales, et ne sauraient faire obstacle ni à l'application des règlements de l'UCI, ni à la compétence du TAS. En juger autrement conduirait à une véritable course à la législation nationale la plus clémente.
42. Il est en effet impératif que les fédérations sportives internationales, telles que l'UCI, aient un droit de regard sur les décisions des fédérations nationales en matière de dopage. Ce droit de regard de l'UCI, qui se matérialise par la possibilité de former un appel à l'encontre des décisions des fédérations nationales devant le TAS, a pour objet de pallier le risque que la compétition internationale ne soit faussée par une fédération nationale qui se garderait de sanctionner ses membres.
43. Par conséquent, l'argument de Monsieur Landaluce tendant à faire valoir que la seule voie de recours contre les décisions du CNCDD serait un appel devant le CESD ne saurait emporter l'adhésion de la Formation.
44. De même, la contestation par Monsieur Landaluce de la compétence du TAS au motif que la réglementation antidopage serait confiée à une autorité publique porte à faux. Il ressort en effet de la jurisprudence du TAS que le pouvoir des fédérations internationales, telles que l'UCI, s'exerce quand bien même le contrôle et la sanction du dopage seraient confiés, comme au cas particulier, à une autorité publique.
45. Le pouvoir des fédérations internationales a pour objet d'éliminer toute concurrence déloyale et toute course à la législation la plus clémente. Elle tend à soumettre tous les athlètes à un traitement égalitaire, en veillant à ce que certaines fédérations nationales ne fassent pas preuve de passivité face aux manquements commis par leurs sportifs nationaux. C'est ainsi que dans une affaire de dopage de nageurs en 1997, le TAS a reconnu comme nécessité impérieuse que les fédérations internationales aient la possibilité de revoir les décisions des fédérations nationales dans les cas de dopage. Il s'agit de prévenir le risque que les compétitions internationales ne soient faussées, en raison des sanctions trop clémentes que pourrait être tentée de prononcer une fédération nationale (TAS 96/156, sentence du 10 octobre 1997, F. / FINA).

46. Par la suite, une autre formation du TAS a eu l'occasion d'étendre cette jurisprudence aux sanctions prononcées par les autorités publiques nationales, dans une sentence B. / Fédération Internationale du Judo du 17 mars 1999 (TAS 98/214, sentence du 17 mars 1999, B. / Fédération Internationale de Judo). Il s'agissait, en l'espèce, d'un contrôle de dopage positif sanctionné par une suspension prononcée par un arrêté du Ministre des Sports français. En effet, une loi française de 1989 donnait audit Ministre le pouvoir de substituer sa décision à toute mesure d'interdiction prise par des fédérations sportives nationales. Au cas particulier, la Fédération française avait prononcé une sanction de deux ans de suspension, dont une avec sursis, à laquelle l'arrêté ministériel avait substitué une simple suspension d'un an. Or, cette dernière sanction n'était pas conforme aux règlements de la Fédération Internationale de Judo (*FIJ*) qui a donc porté l'affaire devant le TAS. Ce dernier a prononcé une suspension de 15 mois.
47. A l'occasion de cette sentence, les arbitres avaient évoqué le problème qui est au cœur du présent litige :

« La formation est d'avis que la latitude accordée par cette jurisprudence aux fédérations internationales doit être étendue aux cas où la procédure de contrôle et la sanction de dopage ne sont pas diligentées par une fédération nationale, conformément à une réglementation sportive, mais par une autorité publique, en application d'une loi nationale, comme en l'espèce, ou, le cas échéant, sur la base d'une convention internationale. »

Le pouvoir d'extension au niveau international des décisions nationales relatives au dopage, quelle que soit l'autorité qui les prononce, se justifie non seulement par le souci de prévenir le risque de voir certaines fédérations ou des organismes gouvernementaux se livrer à une concurrence déloyale des plus malsaines, en omettant de sanctionner leurs sportifs de manière aussi rigoureuse et sévère que d'autres fédérations et / ou que leur fédération internationale, mais aussi par l'objectif que chaque fédération internationale doit chercher à atteindre, à savoir faire respecter un traitement égal et cohérent à tous les pratiquants du même sport ».

48. En conséquence, les dispositions du droit espagnol auxquelles il est fait référence ne permettent pas de conclure à l'incompétence du TAS. C'est ce que le TAS a constaté à différentes reprises, notamment dans le domaine du cyclisme dans l'affaire Muñoz (TAS 2005/A/872, sentence du 30 janvier 2006, UCI / Federico Muñoz Fernandez et Federación Colombiana De Ciclismo), à l'occasion de laquelle la conclusion suivante a été formulée :

“The panel is prepared to accept that as a matter of Colombian Law it was possible for Mr Muñoz to appeal to the General Disciplinary Committee of the Colombian National Olympic Committee. However, to do so was a breach of his contract with the

UCI. At best, the decision of the General Disciplinary Committee could only have an effect within Colombia. It would not entitle Mr Muñoz to participate in cycle races organized under the auspices of the UCI, or to avoid the UCI's disciplinary code”.

49. L'autorité des États et l'autorité sportive internationale ne sont pas en concurrence ; au contraire, leurs rôles sont complémentaires. L'autorité étatique se borne à contrôler la conduite de ses justiciables, tandis que la fédération internationale gère les compétitions qui relèvent de son ressort. Un même comportement peut être sanctionné pénalement dans un lieu donné, sans pour autant entraîner une sanction du cycliste au niveau international. De même, un comportement peut ne pas être sanctionné pénalement tout en étant néanmoins susceptible de générer une exclusion des concours sportifs car il porte atteinte à la loyauté de la compétition.
50. Cette complémentarité entre autorité sportive étatique et internationale peut revêtir une forme particulière lorsqu'une autorité publique se substitue à la fédération nationale pour décider des sanctions – comme ce fut le cas dans l'affaire de la FIJ précitée, ou comme au cas présent. La souveraineté nationale, telle qu'elle s'exprime à l'occasion d'une mesure disciplinaire sportive rendue par une autorité nationale, n'a, en principe, vocation à s'appliquer que sur le seul territoire national. La décision nationale peut toutefois être remplacée par une décision de l'autorité internationale – le TAS – pour que soit assurée la nécessaire uniformité du droit. Certes, il est théoriquement concevable que l'État impose ses décisions nationales jusque dans les compétitions internationales se déroulant sur son territoire au mépris de l'autorité internationale. Un tel comportement irait cependant à l'encontre de tous les efforts tendant à lutter contre le dopage au niveau international, et pourrait conduire à l'exclusion de l'État concerné de l'organisation des compétitions internationales. Il serait surprenant qu'un État souhaite se placer dans une telle situation, et rien dans les textes invoqués dans la présente affaire ne laisse à penser que telle serait la position adoptée par l'Espagne. Bien au contraire, le préambule du Décret Royal 255/1996 du 16 février 1996 reflète clairement que l'Espagne se soucie de la conformité de ses normes avec les normes internationales :
« En application de l'article 76.1.d) de la Loi sur le Sport et en accord avec les critères posés par les normes sportives internationales, le présent Décret Royal identifie les comportements constitutifs d'infractions aux règles du dopage et établit les sanctions y relatives [...] ».
51. Loin de venir au soutien de la prétention de Monsieur Landaluce, le Décret Royal 255/1996 du 16 février 1996 qu'il invoque, ne fait que confirmer le raisonnement qui a été développé ci-avant. En effet, l'article 7 de ce Décret reconnaît explicitement que les

sanctions prononcées par une fédération internationale produisent leurs effets sur l'ensemble du territoire espagnol. Le contraire aurait été pour le moins surprenant.

52. Aux vues de ce qui précède, la Formation conclut qu'elle est compétente pour connaître du présent litige, conformément aux articles 280 à 291 du RCAD.
53. Cela n'est d'ailleurs pas contesté par la RFEC qui a, tout au long de la procédure, reconnu la compétence du TAS et l'applicabilité du RCAD, auquel il était expressément fait référence dans la décision du CNCDD du 5 mai 2006, objet du présent recours.

IV. S'AGISSANT DE LA RECEVABILITÉ DE L'APPEL

54. L'article 285 du RCAD dispose que la déclaration d'appel de l'UCI doit être soumise au TAS dans un délai d'un mois à compter de la réception du dossier complet de l'instance d'audition de la fédération nationale. Toutefois, lorsque l'UCI n'a pas demandé le dossier dans les 15 jours suivant la réception de la décision, comme le prévoit l'article 247 du RCAD, le délai d'appel est d'un mois à compter de la réception de la décision complète.
55. Au cas particulier, l'UCI a reçu la décision du CNCDD le 9 mai 2006 et a demandé le dossier complet le 11 mai 2006. Le dossier complet a été reçu par l'UCI le 26 mai 2006. La déclaration d'appel ayant été envoyée le 26 juin 2006, l'appel a été formé dans les délais.

V. S'AGISSANT DU FOND

A. PRINCIPES GÉNÉRAUX

56. Aux termes de l'article 16 du RCAD :

« La charge de la preuve incombera à l'UCI et à ses fédérations nationales qui devront établir l'existence de la violation d'une règle antidopage. Le degré de preuve établira si l'UCI ou ses fédérations nationales ont satisfait à la charge de la preuve à la satisfaction de l'instance d'audition qui appréciera le sérieux de l'allégation. Le degré de preuve, dans tous les cas, devra être plus important qu'un juste équilibre des probabilités, mais moins qu'une preuve au-delà d'un doute raisonnable. Lorsque le présent règlement antidopage confie au coureur, ou à une autre personne présumée avoir commis une violation des règles antidopage, la charge de renverser une présomption ou d'établir des circonstances ou des faits spécifiques, le degré de preuve devra être fondé sur un juste équilibre de probabilités ».

57. S'agissant du mode d'établissement des faits et présomptions, l'article 18 du RCAD prévoit que :

« Les laboratoires accrédités par l'AMA ou approuvés d'une autre manière par l'AMA sont présumés avoir effectué l'analyse des échantillons et respecté les procédures de la chaîne de sécurité conformément aux standards internationaux pour les laboratoires. Le coureur peut renverser cette présomption en démontrant qu'un écart est survenu par rapport aux standards internationaux.

Si le coureur parvient à renverser la présomption en démontrant qu'un écart est survenu par rapport aux standards internationaux pour les laboratoires, il incombera alors à l'UCI ou à la fédération nationale de démontrer que cet écart n'a pas pu être à l'origine du résultat d'analyse anormal ».

En d'autres termes, il existe une présomption réfragable selon laquelle les laboratoires accrédités par l'AMA ont effectué les analyses dans les règles de l'art, conformément au standard international applicable aux laboratoires (le *SIL*). Dans l'hypothèse où le coureur démontrerait l'existence d'un écart par rapport au *SIL*, il incomberait alors à l'UCI de démontrer que cet écart n'est pas à l'origine du résultat d'analyse anormal. Il convient donc d'appliquer ces règles afin de déterminer si Monsieur Landaluce établit la survenance d'écarts et dans l'affirmative, si l'UCI est en mesure de démontrer que l'écart invoqué n'est pas susceptible d'avoir donné lieu au résultat anormal.

58. Aux termes de l'article 15.1 du RCAD :

« Sont considérées comme violations des règles antidopage:

1. La présence d'une substance interdite, de ses métabolites ou marqueurs dans un prélèvement corporel d'un coureur.

1.1. Il incombe personnellement à chaque coureur de s'assurer qu'aucune substance interdite ne pénètre dans son organisme. Les coureurs sont responsables de toute substance interdite, de ses métabolites ou marqueurs dont la présence est décelée dans leurs prélèvements corporels. Par conséquent, il n'est pas nécessaire de faire la preuve de l'intention, de la faute, de la négligence ou de l'usage conscient de la part du coureur pour établir une violation des règles antidopage en vertu de l'article 15.1 ».

59. La substance testostérone est un stéroïde anabolisant androgène endogène, substance interdite qui figure sur la liste des interdictions 2005 sous la classe S1.1.b. La liste des interdictions prévoit que :

« Dans le cadre d'une substance interdite (selon la liste ci-dessus) pouvant être produite naturellement par le corps, un échantillon sera considéré comme contenant cette substance interdite si la concentration de la substance interdite ou de ses métabolites ou de ses marqueurs et/ou tout autre rapport pertinent dans l'échantillon du sportif s'écarte suffisamment des valeurs normales trouvées chez l'homme pour qu'une production endogène normale soit improbable. Un échantillon ne sera pas considéré comme contenant une substance interdite si le sportif prouve que la concentration de substance interdite ou de ses métabolites ou de ses marqueurs et/ou tout autre rapport pertinent dans l'échantillon du sportif est attribuable à un état physiologique ou pathologique. Dans tous les cas, et quelle que soit la concentration, le laboratoire rendra un résultat d'analyse anormal si, en se basant sur une méthode d'analyse fiable, il peut démontrer que la substance interdite est d'origine exogène.

Si le résultat de laboratoire n'est pas concluant et qu'aucune concentration décrite au paragraphe ci-dessus n'est mesurée, l'organisation antidopage responsable effectuera une investigation plus approfondie s'il existe de sérieuses indications, telles que la comparaison avec des profils stéroïdiens de référence, d'un possible usage d'une substance interdite.

Si le laboratoire a rendu un rapport T/E supérieur à quatre (4) pour un (1) dans l'urine, une investigation complémentaire est obligatoire afin de déterminer si le rapport est dû à un état physiologique ou pathologique, sauf si le laboratoire rapporte un résultat d'analyse anormal basé sur une méthode d'analyse fiable, démontrant que la substance interdite est d'origine exogène ».

B. APPLICATION DES PRINCIPES GÉNÉRAUX AU CAS PARTICULIER

60. Au cas particulier, le LNDD a utilisé la méthode d'analyse GC/C/IRMS, analyse par spectrométrie de masse du rapport isotopique. Le rapport d'analyse du LNDD a révélé la présence de testostérone de nature exogène. L'échantillon A présente un ratio T/E de 9,2, supérieur à la limite de 4 établie par l'AMA. Le rapport d'analyse du LNDD relatif à l'échantillon B confirme le résultat de l'analyse de l'échantillon A.
61. Le LNDD ayant effectué des analyses dont le résultat est positif, il appartient à Monsieur Landaluce de démontrer qu'un écart est survenu par rapport au SIL pour renverser la présomption selon laquelle ces analyses ont été réalisées dans les règles de l'art. Comme l'a rappelé à juste titre l'UCI au cours de l'Audience, Monsieur Landaluce ne pourrait se contenter d'indiquer que *« something could potentially be wrong »*, mais doit bien rapporter la preuve d'une violation du SIL.

62. Pour ce faire, Monsieur Landaluce conteste la validité des analyses pratiquées par le LNDD, invoquant des manquements et des irrégularités commis lors du processus d'analyse. Il fait valoir que ces irrégularités rendaient « *invalides* » les résultats de l'analyse effectuée et a indiqué que « *ces résultats ne seront jamais considérés comme preuve de la commission d'une faute pouvant être sanctionnée* ».
63. A cette fin, Monsieur Landaluce souligne qu'il fait siennes les observations du Dr de Boer et de Madame Luisa Lurueña Sánchez, pharmacienne et spécialiste en analyse et contrôle des médicaments et drogues.
64. Madame Luisa Lurueña Sánchez observe que :
- « Avec la documentation remise nous pouvons dire que celle-ci est insuffisante pour démontrer l'abus de testostérone ou un de ses précurseurs.
Etant donnée la mauvaise, faible et inconsistante documentation analytique réalisée apportée pour démontrer l'abus de testostérone ou analogues dans cet échantillon, on considère que l'innocence du sportif peut être défendue ».*
65. Au soutien de sa prétention, Monsieur Landaluce a également versé aux débats de nombreux rapports élaborés par le Dr de Boer qui estime que plusieurs irrégularités auraient été commises par le LNDD et souligne que le SIL n'aurait pas été respecté. Dans son rapport du 12 mars 2006, le Dr de Boer observe notamment que :
- « Le laboratoire AMA de Châtenay-Malabry n'a pas fait d'erreur concernant l'analyse chimique, mais il n'a pas respecté, sous divers aspects, les normes internationales pour les laboratoires d'analyse (ISL, International Standards for Laboratories) de l'AMA. Ainsi, plusieurs non-conformités se sont produites, ce qui, entre autres, a abouti au fait que l'analyse des échantillons « A » et « B » était loin d'être complète ».*
66. Monsieur Landaluce invoque six écarts qui auraient été commis par le laboratoire par rapport aux normes du SIL. Quatre des manquements allégués ont trait à l'appréciation scientifique qui a été faite par le laboratoire, tandis que les deux autres sont relatifs à la procédure mise en œuvre par lui. La Formation se doit d'examiner les prétendues irrégularités invoquées par Monsieur Landaluce afin de déterminer si le LNDD s'est conformé au SIL.

1. S'agissant des manquements allégués ayant trait à l'appréciation scientifique par le LNDD

- a) L'argument selon lequel le dossier ne permettrait pas à un autre analyste d'évaluer les analyses réalisées et d'en interpréter les données (point 5.2.6.1 du SIL)*

67. Aux termes du point 5.2.6.1 du SIL :

«Le Laboratoire doit disposer de procédures documentées assurant la tenue coordonnée d'un dossier relatif à chaque Échantillon analysé. Dans le cas d'un Résultat d'analyse anormal, ce dossier doit comprendre les données justifiant les conclusions présentées (voir Document Technique "Documentation du Laboratoire"). En règle générale, le dossier doit être tenu de telle sorte que, en l'absence de l'analyste, un autre analyste compétent puisse évaluer les analyses réalisées et en interpréter les données ».

68. Le Dr de Boer a déclaré qu'il n'avait pas pu évaluer différents aspects des analyses en raison d'un manque d'information et a indiqué que les rapports relatifs aux échantillons A et B ne contenaient pas les informations permettant d'identifier les stéroïdes impliqués.

69. Le Dr Saugy a, pour sa part, indiqué que les informations permettaient d'identifier les stéroïdes impliqués. En effet, dans sa déclaration du 14 juillet 2006, le Dr Saugy a indiqué qu' *« après lecture attentive et raisonnable des paquets de documentation, j'estime que tous les aspects permettant de faire une évaluation du dossier sont présents dans celui-ci ».*

70. La Formation constate l'existence d'un désaccord entre les experts. Tandis que le Dr Saugy estime que le dossier permettait d'identifier les stéroïdes impliqués, le Dr de Boer considère quant à lui que les éléments dont il disposait étaient insuffisants. Les arbitres considèrent que le témoignage du Dr Saugy est plausible et que la démonstration faite par le Dr de Boer ne parvient pas à invalider son analyse.

71. En conséquence, la Formation estime que Monsieur Landaluce n'a pas démontré l'existence d'une violation du point 5.2.6.1 du SIL et qu'il n'est donc pas parvenu à renverser la présomption selon laquelle l'analyse du LNDD avait été réalisée dans les règles de l'art, telles qu'elles résultent du point 5.2.6.1 du SIL.

- b) *L'argument selon lequel la substance analysée serait une substance à seuil et que l'incertitude de 0,8 adoptée par le LNDD serait insuffisante (point 5.4.4.1.3 du SIL)*

72. Aux termes du point 5.4.4.1.3 du SIL :

« Le Laboratoire sera tenu d'atteindre, tant pour les Substances sans seuil que pour les Substances à seuil, la Limite Minimale de Performance Requise définie pour la détection et l'identification de la substance ou pour la démonstration de sa présence au-delà du seuil toléré (si nécessaire) ».

73. Le Dr de Boer estime que la valeur de l'incertitude de 0,8 présentée par le LNDD serait insuffisante et devrait être de 1,35. Il indique par ailleurs que le fait qu'un indice d'incertitude ait été donné démontrerait que le LNDD aurait considéré être en présence d'une substance à seuil dans la mesure où l'incertitude ne serait prise en compte que pour les substances à seuil. Le Dr de Boer conclut à l'existence d'un écart par rapport au point 5.4.4.1.3 du SIL.

74. Le Prof. de Ceaurriz et le Dr Saugy estiment quant à eux que cette valeur, utilisée par de nombreux laboratoires, serait conforme au point 5.4.4.1.3 du SIL. Dans sa déclaration du 14 juillet 2006, le Dr Saugy considère qu'il ne s'agit pas d'une mesure d'une substance à seuil mais d'une méthode de confirmation basée sur des mesures quantitatives, dont le but est de démontrer l'origine qualitative du produit ingéré.

75. L'UCI fait valoir qu'il ne s'agit pas d'une substance à seuil dans la mesure où la simple présence de testostérone exogène suffit. L'analyse isotopique viserait uniquement à démontrer la nature exogène du produit, sans se préoccuper de sa quantité.

76. Au soutien de son allégation, l'UCI se réfère au point 5.4.4.3 du SIL qui indique que :

« Dans la plupart des cas, l'identification d'une substance interdite ou des métabolite(s) ou marqueur(s) associés suffit pour que soit déclaré un résultat d'analyse anormal. La notion d'incertitude quantitative définie dans l'ISA/CEI 17025 n'est donc pas applicable ici. [...] »

Dans le cas des substances à seuil, il faut considérer à la fois l'incertitude sur l'identification et l'incertitude sur la démonstration de la présence de la substance à concentration supérieure au seuil ».

77. Dans sa déclaration du 14 juillet 2006, le Dr Saugy observe qu'au demeurant, le document technique TD2004 EAAS de l'AMA indiquerait que le résultat démontre l'administration d'un stéroïde exogène lorsque la valeur du métabolite diffère significativement de 3‰ ou plus de la valeur de la référence endogène utilisée.

Le Dr Saugy déclare qu'au cas particulier, l'analyse aurait révélé, tant pour l'échantillon A que pour l'échantillon B, une différence significativement supérieure à 3 ‰, à savoir 4,85 ‰ pour l'échantillon A et 4,76 ‰ pour l'échantillon B, entre la référence endogène et le métabolite retrouvé dans la testostérone.

78. La Formation constate qu'il n'existe en la matière aucune méthode précise et que les experts présents lors de l'Audience ne se sont pas accordés sur une valeur d'incertitude à prendre en compte pour l'analyse isotopique.
79. La Formation estime que Monsieur Landaluce n'a pas renversé la présomption selon laquelle le LNDD s'est conformé aux règles de l'art. En effet, les experts cités par Monsieur Landaluce, s'ils ont démontré leur désaccord avec la méthode de calcul adoptée par le LNDD, n'ont pas pour autant renversé la présomption selon laquelle les analyses se sont conformées au point 5.4.4.1.3 du SIL. La Formation estime donc que Monsieur Landaluce n'a pas rapporté la preuve d'un écart par rapport au point 5.4.4.1.3 du SIL.

c) L'argument selon lequel les informations sollicitées n'auraient pas été remises au cycliste ni à son expert (point 5.4.7.3 du SIL et des documents techniques TD2004EAAS et TD2003IDCR)

80. Monsieur Landaluce fait valoir qu'il n'aurait pas reçu les informations sollicitées à plusieurs reprises, tant par lui que par le Dr de Boer, qui auraient permis de procéder à l'évaluation de l'analyse effectuée par le LNDD.
81. L'UCI a indiqué qu'un courrier de la RFEC du 22 novembre 2005 démontrerait que Monsieur Landaluce a reçu l'intégralité des documents relatifs à l'analyse des échantillons A et B. L'UCI a également fait valoir qu'un courrier aurait été envoyé directement par le LNDD à la RFEC contenant les informations requises. Ce courrier aurait bien été reçu par la RFEC qui aurait tamponné cette correspondance, bien que la décision du CNCDD indique qu'aucune réponse de la LNDD n'aurait été reçue.
82. Par ailleurs, le Dr de Boer indique avoir reçu le 29 août 2006 un document contenant l'évaluation des temps de rétention concernant l'identification des stéroïdes impliqués dans l'analyse de l'échantillon B. Il observe toutefois qu'aucune information ne lui aurait été transmise s'agissant des données de masse spectrale et d'identification des stéroïdes impliqués dans l'analyse de l'échantillon A. Le Dr de Boer estime en

conséquence qu'il existerait un écart par rapport au point 5.4.7.3 du SIL et aux documents techniques TD2004EAAS et TD2003IDCR.

83. La Formation constate une nouvelle fois l'existence d'une divergence d'opinion entre les experts présents lors de l'Audience. Le Dr Saugy indique en effet que les informations nécessaires à l'identification de ces produits auraient été fournies par le LNDD. Cette analyse qui émane d'un expert jouissant d'une expérience non négligeable dans ce domaine est tout à fait plausible. Par conséquent, la Formation considère que Monsieur Landaluce n'a pas renversé la présomption selon laquelle le LNDD se serait conformé au point 5.4.7.3 du SIL et aux documents techniques TD2004EAAS et TD2003IDCR.

d) Les arguments selon lesquels les valeurs ne diffèreraient pas de manière significative de trois unités delta ou plus et qu' il aurait fallu analyser trois aliquotes (document technique TD2004EAAS)

84. Aux termes du document technique TD2004EAAS :

« Les résultats seront rapportés comme étant cohérents avec l'administration d'un stéroïde, lorsque la valeur 13C/12C mesurée pour le(s) métabolite(s) diffère de manière significative, à savoir d'au moins 3 unités delta, du stéroïde de référence urinaire sélectionné. Dans certains Echantillons, la mesure de la valeur 13C/12C du/des stéroïde(s) de référence sélectionné(s) peut ne pas être possible du fait de concentration(s) basse(s). Les résultats de telles analyses seront rapportés comme « non concluants », sauf si le rapport mesuré du (des) métabolite(s) est inférieur à - 28 ‰ sur la base du stéroïde non dérivé ».

85. D'après le Dr de Boer, ce document technique imposerait que les valeurs diffèrent de manière significative de trois unités delta ou plus. Par ailleurs, s'agissant d'une valeur seuil, le SIL imposerait l'analyse de trois aliquotes ou, dans l'hypothèse où, comme au cas particulier, un seul aliquote serait disponible, une triple analyse de celui-ci.
86. Le Prof. de Ceaurriz et le Dr Saugy estiment pour leur part qu'une mesure en triplicat de l'échantillon n'est pas nécessaire et qu'une telle exigence, qui n'existerait pas au demeurant, serait au contraire problématique dans la mesure où le volume d'urine nécessaire ne serait pas disponible. Ils indiquent par ailleurs, qu'il ne s'agirait pas d'une mesure à seuil nécessitant l'analyse de plusieurs échantillons.
87. La Formation constate, ici aussi, l'existence d'une divergence d'appréciation entre les experts et considère que Monsieur Landaluce n'a pas renversé la présomption selon laquelle le LNDD s'est conformé au document technique TD2004EAAS.

2. S'agissant des manquements allégués ayant trait à la procédure suivie par le LNDD

a) *L'argument selon lequel les délais n'auraient pas été respectés (points 5.2.4.3.2.1 et 5.2.6.5 du SIL)*

88. Le Dr de Boer fait valoir que le LNDD n'aurait pas respecté les délais prévus par les points 5.2.4.3.2.1 et 5.2.6.5 du SIL.

89. Aux termes du point 5.2.4.3.2.1 du SIL :

« Dans les cas où est demandée une analyse de confirmation sur l'Échantillon "B" de la présence d'une Substance interdite, de Métabolite(s) associé(s) ou de Marqueur(s) indiquant l'Usage d'une Substance interdite ou d'une Méthode interdite, l'analyse de l'Échantillon "B" doit intervenir dès que possible et être conclue dans les trente (30) jours suivant la notification du Résultat d'analyse anormal pour l'Échantillon "A" ».

90. Le point 5.2.6.5 du SIL exige que :

« Les résultats concernant l'Échantillon "A" devront être rendus dans les dix (10) jours ouvrables à compter de la réception de l'Échantillon. [...] Le délai pourra être modifié par accord mutuel entre le Laboratoire et l'Autorité de contrôle ».

91. Le Dr de Boer souligne que les résultats de l'échantillon A, qui auraient dû être rendus dans les 10 jours ouvrables à compter du 14 juin 2005, date de la réception dudit échantillon par le LNDD, ne l'auraient été que 23 jours plus tard, soit le 17 juillet 2005. Le Dr de Boer indique également que l'analyse de l'échantillon B n'aurait pas été réalisée dans le délai imparti de trente jours suivant la notification du résultat d'analyse anormal de l'échantillon A. En effet, la notification du résultat de l'analyse A a eu lieu le 28 juillet 2005. Le LNDD a proposé la date du 11 septembre 2005 pour l'ouverture de l'échantillon B, date qui, à la demande de Monsieur Landaluce, a été reportée au 21 septembre 2005, puis au 19 octobre 2005.

92. L'UCI a reconnu que les délais n'avaient pas été respectés, invoquant « *la surcharge des laboratoires, la complexité de l'analyse et la période des vacances* ».

93. Au cours de l'Audience, l'ensemble des experts présents a déclaré que les délais n'avaient eu aucun effet sur les résultats des analyses.

94. En application des règles relatives aux modes d'établissement des faits et présomptions, la Formation constate que Monsieur Landaluce est parvenu à renverser la présomption selon laquelle le LNDD se serait conformé aux points 5.2.4.3.2.1 et 5.2.6.5 du SIL en démontrant l'existence d'un écart. Une telle démonstration n'a toutefois qu'un effet

purement théorique, l'ensemble des experts présents à l'Audience ayant observé que l'écart constaté ne pouvait être à l'origine du résultat positif de l'analyse des échantillons A et B. La Formation conclut par conséquent que le résultat anormal de Monsieur Landaluce ne résulte pas de l'écart par rapport aux points 5.2.4.3.2.1 et 5.2.6.5 du SIL.

b) *L'argument selon lequel l'analyste qui a effectué l'analyse de l'échantillon B était également impliquée dans l'analyse de l'échantillon A (point 5.2.4.3.2.2 du SIL)*

95. Aux termes du point 5.2.4.3.2.2 du SIL :

« La confirmation sur l'Échantillon "B" doit être réalisée dans le même Laboratoire que celle effectuée sur l'Échantillon "A", par un analyste différent. La (les) opérateurs qui ont effectué l'analyse "A" peuvent toutefois procéder aux réglages instrumentaux, aux contrôles de conformité et à la vérification des résultats ».

96. Monsieur Landaluce se fonde sur le rapport du Dr de Boer pour faire valoir que l'analyste qui a mené l'analyse de l'échantillon B était également impliquée dans l'analyse de l'échantillon A, en violation du point 5.2.4.3.2.2 du SIL.

97. Le Dr de Boer a indiqué que cette norme interdit qu'un même analyste touche/manipule à la fois l'échantillon A et l'échantillon B (*« touching the sample must be separate »*).

98. Au cas particulier, le rapport du 11 juin 2005 révèle que l'analyste qui a procédé à l'analyse de l'échantillon B a, dans le cadre de l'analyse A, réalisé les tâches suivantes : *package* à 4°C, reprise par acétonitrile et mise en vial, évaporation, reprise par hexane et injection CG/MS.

99. Le Président de la Formation a demandé au Dr Saugy si le point 5.2.4.3.2.2 du SIL interdisait que le même analyste touche/manipule à la fois les échantillons A et B. Le Dr Saugy a acquiescé dans les termes suivants : *« je suis d'accord que ça exclut toute manipulation de l'échantillon »*.

100. Le Président de la Formation a ensuite interrogé le Prof. de Ceaurriz afin de savoir si le même analyste avait touché/manipulé les échantillons A et B. Le Prof. de Ceaurriz a répondu :

« Oui. C'est clairement indiqué. Si vous voulez c'est indiqué dans notre chaîne de [sécurité]. Il n'y a pas d'ambiguïté là-dessus. [L'analyste] a touché les échantillons dans le A et a touché la totalité des échantillons dans le B. Il n'y a pas d'ambiguïté là-dessus ».

101. Le Président de la Formation a alors demandé si cela était constitutif d'un écart par rapport au SIL, ce à quoi le Prof. de Ceaurriz a répondu :

« Tout à fait. C'est même ouvertement dans les documents du laboratoire. [...] par rapport à la norme, effectivement, elle a eu un contact avec l'échantillon ».

102. Le Prof. de Ceaurriz a indiqué qu'il y avait eu 10 % de « *chevauchement pour des raisons de charge de travail entre deux personnes* ».

103. L'ensemble des experts présents à l'Audience a donc reconnu que l'analyste qui est intervenue dans le cadre des deux analyses ne s'était pas bornée à « *procéder aux réglages instrumentaux, aux contrôles de conformité et à la vérification des résultats* » et qu'un écart était constaté par rapport au point 5.2.4.3.2.2 du SIL.

104. En application des règles relatives aux modes d'établissement des faits et présomptions, la Formation constate que Monsieur Landaluce parvient une nouvelle fois à renverser la présomption selon laquelle le LNDD se serait conformé au point 5.2.4.3.2.2 du SIL, en démontrant l'existence de cet écart. Les conséquences sont cette fois décisives.

105. Il n'a pas été démontré que cela n'était pas à l'origine du résultat anormal, ni d'ailleurs que cela l'était. Il incombait toutefois à l'UCI, aux termes de l'article 18 du RCAD, de démontrer que l'écart par rapport au SIL n'était pas à l'origine de l'analyse anormale, ce qui n'a pas été fait. L'UCI s'est contentée d'indiquer dans son mémoire d'appel que :

« Et même si écart il y aurait eu – quod non – celui-ci n'a pu conduire au résultat d'analyse anormal, sauf s'il est établi que [l'analyste] avait commis une erreur ayant causé le résultat d'analyse anormal, quod non ».

106. De même lors de l'Audience, l'UCI a simplement observé :

« Quant aux écarts avec le standard qui ont été signalés, je crois pouvoir conclure que s'il y en a eu, ils ne sont pas significatifs et ne sont certainement pas à l'origine du résultat ».

107. Or, il appartenait bien à l'UCI de démontrer que le non-respect du point 5.2.4.3.2.2 du SIL n'était pas à l'origine du résultat anormal. Dans la mesure où l'UCI n'est pas parvenue à ce faire, la Formation ne peut que conclure à la mise hors de cause de Monsieur Landaluce.

108. Il est vrai qu'une sentence ayant eu à se prononcer sur cette question de la double intervention d'un même analyste dans le cadre de l'examen des échantillons A et B, dans l'affaire Wang Lu Na et autres contre FINA (TAS 98/208, sentence du 22 décembre 1998, Wang Lu Na et autres / FINA), a confirmé la suspension de quatre nageuses.

L'apport de cette sentence est toutefois très marginal pour les besoins de la présente affaire, et ce pour trois raisons. Premièrement, la réglementation applicable est différente. Ensuite, la sentence indique expressément (*paragraphe 5.22*) que le rôle joué par les analystes concernés n'était pas clairement établi : les arbitres ont déclaré qu'ils étaient « *unpersuaded* » par l'argument selon lequel les analystes « *were involved in anything other than the tests of the controls* » par opposition aux « *A samples themselves* ». Or, au cas particulier, comme cela a été démontré précédemment, il y a identité des analystes ayant manipulé les échantillons A et B. Enfin, les représentants des nageuses avaient, par leurs signatures, reconnu que la procédure prévue par le règlement de la FINA avait été respectée par le laboratoire. En l'espèce, l'UCI ne s'est pas attachée à rapporter la preuve de ce que Monsieur Landaluce avait approuvé la procédure suivie.

109. Bien que conscient des impératifs de coûts et d'organisation auxquels sont confrontés les laboratoires, la Formation se doit de veiller au respect de règles fondamentales, compte tenu des implications que sa décision pourrait avoir sur la réputation, et partant, la carrière de l'athlète, si une sanction disciplinaire était prononcée à son encontre.
110. La Formation n'est pas sans savoir que la norme qui impose qu'un analyste différent procède à l'analyse de l'échantillon B a fait l'objet de discussions vives entre l'AMA et les responsables de laboratoires. Ces derniers font valoir que cette règle complique déraisonnablement le fonctionnement des laboratoires, sans pour autant qu'il soit démontré qu'elle apporte une protection supplémentaire des athlètes contrôlés. Il serait assurément irréaliste d'exiger qu'un même analyste effectue l'intégralité d'une analyse du début à la fin. En effet, les analyses peuvent, lorsqu'elles portent sur certaines substances, durer plusieurs jours au cours desquels des procédés sont machinalement mis en œuvre. Les analystes effectuent de nombreuses tâches, passant de l'une à l'autre, afin que plusieurs analyses puissent être réalisées simultanément. S'il est envisageable d'exiger d'un grand laboratoire comptant un effectif de 50 à 100 personnes, que le travail y soit organisé de façon à exclure de l'analyse de l'échantillon B l'analyste qui aurait effectué l'analyse de l'échantillon A – bien que cela constitue un facteur de complication non négligeable dont les laboratoires souhaiteraient se dispenser – une telle exigence constituerait un facteur de complication majeur pour un laboratoire de moindre taille.

111. Il est quasiment impossible de prouver un fait négatif, soit en l'occurrence que l'intervention du même analyste dans le cadre des deux analyses n'a pas pu affecter le résultat. Certains responsables de laboratoire estiment par conséquent que cette règle serait trop rigide ; en réalité, une protection suffisante des athlètes est déjà assurée dans la mesure où le système d'identification des échantillons par codes permet de s'assurer de ce que leur identité n'est pas connue des analystes.
112. Ce raisonnement, bien que rationnel et plausible, échoue devant le TAS pour une raison très simple : les arbitres ne créent pas les règles, ils les appliquent. Cela est d'autant plus vrai que les auteurs du règlement antidopage ont conservé la règle qui impose qu'un autre analyste soit chargé de l'analyse de l'échantillon B, alors même qu'ils avaient entendu les observations des responsables de laboratoires. Les règles peuvent certes être modifiées ou affinées, mais tel n'est pas le rôle du TAS.
113. La règle applicable est claire et dépourvue de toute souplesse. Les arbitres du TAS n'ont pas pour mission de modifier les règles ni vocation à s'approprier un pouvoir discrétionnaire lorsque aucun texte ne les autorise à le faire.
114. L'on notera en passant que si les cas où le TAS conclut à la mise hors de cause d'un athlète dans l'hypothèse où un laboratoire n'aurait pas respecté les protocoles sont peu fréquents, cela s'explique par le fait que les autorités disciplinaires connaissent la sévérité des arbitres à cet égard et renoncent à poursuivre les athlètes – même en présence de cas très suspects – lorsque les analyses n'ont pas été réalisées dans le respect des règles de l'art.
115. La Formation tient à souligner la bonne foi du personnel du LNDD qui n'est pas en cause. Les arbitres n'ont aucune raison de douter de l'explication fournie par le Prof. De Ceaurriz selon laquelle le « *chevauchement* » des opérations effectuées par les analystes était dû à une surcharge de travail au sein du LNDD. Il a par ailleurs fait observer que des personnes malveillantes et complices auraient couvert leurs agissements en rédigeant les rapports relatifs aux analyses de façon à ce qu'aucun manquement éventuel ne puisse leur être reproché. Au cas présent, c'est tout le contraire ; l'athlète obtient gain de cause sur la base des informations dont disposait le laboratoire et qui ont été loyalement communiquées.
116. Il convient encore de préciser que les paragraphes 95 à 115 de cette décision ont été adoptés à la majorité de la Formation arbitrale, selon l'article R59 du Code, et que

l'opinion minoritaire de l'arbitre dissident a été dûment considérée et débattue lors des délibérations.

117. En tout état de cause, la présente sentence ne constitue pas une déclaration d'innocence de Monsieur Landaluce au regard des règles antidopage. Monsieur Landaluce bénéficie simplement d'une règle formelle et pourtant fondamentale, tendant à garantir les droits des personnes soumises à des contrôles antidopage.

VI. S'AGISSANT DES FRAIS

118. En ce qui concerne les litiges disciplinaires à caractère international jugés en appel, tel que celui qui nous occupe, l'article R65 du Code prévoit ce qui suit:

« Sous réserve des articles R65.2 et R65.4, la procédure est gratuite.

Les frais et honoraires des arbitres, calculés selon le barème du TAS, ainsi que les frais du TAS sont à la charge du TAS.

Lors du dépôt de la déclaration d'appel, l'appelant verse un droit de Greffe minimum de CHF 500.—, faute de quoi le TAS ne procède pas et l'appel est réputé retiré. Cet émolument reste acquis au TAS.

Les frais des parties, témoins, experts et interprètes sont avancés par les parties. La Formation en attribue la charge dans la sentence en tenant compte du résultat de la procédure, du comportement et des ressources financières des parties.

Si l'ensemble des circonstances le justifie, le Président de la Chambre arbitrale d'appel peut, d'office ou sur demande du Président de la Formation, appliquer les articles R64.4 et R64.5, 1^{ère} phrase, à une procédure arbitrale d'appel ».

119. Les articles R64.4 et R64.5 du Code prévoient ce qui suit :

« A la fin de la procédure, le Greffe arrête le montant définitif des frais de l'arbitrage qui comprennent le droit de Greffe du TAS, les frais administratifs du TAS calculés selon le barème du TAS, les frais et honoraires des arbitres calculés selon le barème du TAS, une participation aux débours du TAS et les frais de témoins, experts et interprètes. Le décompte final des frais de l'arbitrage peut soit figurer dans la sentence, soit être communiqué aux parties séparément ».

« La sentence arbitrale détermine quelle partie supporte les frais de l'arbitrage ou dans quelle proportion les parties en partagent la charge. La sentence condamne en principe la partie qui succombe à une contribution aux frais d'avocat de l'autre partie, ainsi qu'aux frais encourus par cette dernière pour les besoins de la procédure, notamment les frais de témoins et d'interprète. Lors de la condamnation aux frais d'arbitrage et d'avocat, la Formation tient compte du résultat de la procédure, ainsi que du comportement et des ressources des parties ».

120. En l'espèce, l'appel de l'UCI est rejeté et la décision dont appel doit être confirmée. Cet arbitrage étant gratuit pour les parties, dès lors qu'il concerne un litige de nature

disciplinaire à caractère international, il s'agit de déterminer si et dans quelle mesure Monsieur Landaluce et la RFEC ont droit à l'octroi d'une indemnité pour leurs frais d'avocat et pour les autres frais relatifs à la présente procédure d'arbitrage. Après avoir dûment considéré l'ensemble des circonstances de l'affaire, notamment le grand nombre d'arguments futiles invoqués par les défendeurs, la Formation considère qu'il est équitable de laisser à la charge de chacune des parties les frais encourus dans le cadre de cette procédure.

VII. S'AGISSANT DE LA CONFIDENTIALITE

121. Aux termes de l'article R59 alinéa 6 du Code de l'arbitrage en matière de sport :

« La sentence, un résumé et/ou un communiqué de presse faisant état de l'issue de la procédure est publié par le TAS, sauf si les parties conviennent que l'arbitrage doit rester confidentiel ».

122. Monsieur Landaluce a indiqué qu'il souhaitait que la procédure demeure confidentielle et qu'aucun communiqué de presse faisant état de la procédure ne soit publié par le TAS.

123. L'UCI a indiqué que la procédure initiée à l'encontre de Monsieur Landaluce était d'ores et déjà connue du public, celle-ci ayant fait l'objet d'un communiqué de presse par le TAS.

124. Lors de l'Audience, l'UCI, Monsieur Landaluce et la RFEC ne sont pas parvenus à un accord concernant le caractère confidentiel ou non de la présente procédure. Dans ces circonstances, le TAS pourra, s'il le juge opportun, publier un résumé et/ou un communiqué de presse faisant état de l'issue de la présente procédure.

* * * * *

PAR CES MOTIFS

Le Tribunal Arbitral du Sport prononce :

1. Le Tribunal Arbitral du Sport est compétent pour connaître de l'appel interjeté par l'Union Cycliste Internationale.
2. L'appel formé le 26 juin 2006 par l'Union Cycliste Internationale contre la décision rendue le 5 mai 2006 par le Comité Nacional de Competición y Disciplina Deportiva de la Real Federación Española de Ciclismo est rejeté.
3. La sentence est rendue sans frais, à l'exception de l'émolument de greffe de CHF 500 versé en début de procédure par l'Union Cycliste Internationale, qui reste acquis au TAS.
4. Chaque partie garde ses frais d'avocat et autres frais découlant du présent arbitrage.

Lausanne, le 19 décembre 2006

Le TRIBUNAL ARBITRAL DU SPORT

Le Président de la Formation

Jan Paulsson

Olivier Carrard
Arbitre

José Juan Pintó
Arbitre

Shaparak Saleh
Greffier ad hoc

**TAS 2006/A/1119 Union Cycliste Internationale (UCI) v. Iñigo Landaluze Intxaurreaga &
Real Federación Española de Ciclismo (RFEC)**

ARBITRAL AWARD

delivered by the

COURT OF ARBITRATION FOR SPORT

sitting in the following composition:

President: Jan Paulsson, Attorney-at-law, Paris, France

Arbitrators: Olivier Carrard, Attorney-at-law, Geneva, Switzerland
José Juan Pintó, Attorney-at-law, Barcelona, Spain

Ad-hoc Clerk: Shaparak Saleh, Attorney-at-law, Paris, France

in the arbitration between

The Union Cycliste Internationale (UCI), Aigle, Switzerland
Represented by Philippe Verbiest, Attorney-at-law, Leuven, Belgium

versus

Iñigo Landaluze Intxaurreaga, Spain
Represented by Carmen Coello de Portugal Silva and Immaculada Herranz Perlado, Attorneys-
at-law, Madrid, Spain

and

Real Federación Española de Ciclismo (RFEC), Madrid, Spain
Represented by José Antonio del Valle Herán, Attorney-at-law, Madrid, Spain

* * * * *

I. FACTS

1. The Union Cycliste Internationale (**UCI**) is the association of national cycling federations. The purposes of the UCI are to direct, develop, regulate, control and discipline cycling. The antidoping system put in place by the UCI includes the Antidoping Rules (**ADR**), the list of prohibited doping classes of substances and methods, the list of international events subject to doping control and the list of accredited laboratories.
2. The Real Federación Española de Ciclismo (**RFEC**) is the Spanish national cycling federation. It has disciplinary regulations at the national level and is a member of the UCI.
3. Mr. Iñigo Landaluze Intxaurreaga (**Mr. Landaluze**) is a Spanish rider in the elite category, holder of a license granted by the RFEC. At the time of the facts, he was a member of the Euskaltel cycling team and participated in the international road cycling race "Critérium du Dauphiné Libéré" which took place from June 5 to 12, 2005.
4. At the end of the June 11, 2005 stage, Mr. Landaluze underwent a doping control test. During sample collection, Mr. Landaluze indicated that he had taken triamcinolone by infiltration on March 21, 2005.
5. The A sample collected from Mr. Landaluze was analyzed by the French antidoping lab in Châtenay-Malabry (**LNDD**), a laboratory accredited by the World Anti-Doping Agency (**WADA**). Its analytical report dated July 17, 2005 revealed that *"the isotopic ratio mass spectrometric analysis indicates that Testosterone or one of its precursors was taken."* On July 28, 2005, the analytical report for the A sample was communicated to the RFEC, so that disciplinary proceedings would take place, in accordance with article 187 of the ADR.
6. On August 11, 2005, Mr. Landaluze requested, via the RFEC, a B sample analysis. He then asked that the B sample analysis, which was originally scheduled for September 14 and 21, 2005, be postponed to October 19, 2005. On October 21, 2005, the B sample analysis confirmed the presence of exogenous testosterone in the B sample.
7. By a fax dated October 27, 2005, the UCI communicated the B sample analysis results to the RFEC and asked the latter to proceed with the disciplinary proceedings.
8. On May 5, 2006, the RFEC, via the Comité Nacional de Competición y Disciplina Deportiva (national committee for competition and sport discipline/**CNCDD**) deemed that the conditions in which the analysis had been done revealed the existence *"of an incomplete process not in conformity with all applicable legal requirements which, therefore, could not totally guarantee the result."* The CNCDD determined that *"the legal principle, 'in dubio pro reo' [giving the defendant the benefit of the doubt], [was] fully applicable to the present case,"* shelved the case and acquitted Mr. Landaluze.
9. Mr. Landaluze did not participate in any cycling race between July 24, 2005 and May 15, 2006.

II. PROCEEDINGS

A. PARTIES' SUBMISSIONS

10. The UCI brought the case before the Court of Arbitration for Sport (**CAS**) in a statement dated June 26, 2006.

On July 17, 2006, the UCI filed its appeal brief requesting that the CAS:

- "1) overturn the decision of the RFEC national committee for competition and sport discipline;*
- 2) suspend Mr. Landaluce Intxaurreaga for 2 years, in accordance with the ADR;*
- 3) disqualify Mr. Landaluce Intxaurreaga from cycling races in which he participated beginning June 11, 2005, including in particular the cycling race "Critérium du Dauphiné Libéré", until at least July 29, 2005.*
- 4) order Mr. Landaluce Intxaurreaga and the Real Federación Española de Ciclismo to pay the UCI the sum of 1,000 Swiss francs for the costs of managing the results (ADR article 245.2).*
- 5) order Mr. Landaluce Intxaurreaga and the Real Federación Española de Ciclismo to reimburse the UCI for the fee of 500 Swiss francs and all other costs, including a contribution to the UCI costs."*

11. Mr. Landaluce filed his response on September 8, 2006, requesting that the CAS:

- declare that it has no jurisdiction on this case;
- order the UCI to pay the costs generated by the proceedings.

Mr. Landaluce made the subsidiary request that the CAS:

- dismiss the appeal filed by the UCI;
- order no sanction against him;
- order the UCI to pay costs.

Mr. Landaluce made the even more subsidiary claim that any sanction against him would have to be reduced by nine and a half months, the period during which he abstained from racing.

12. The RFEC filed its response on September 14, 2006, essentially claiming that:

- the CNCDD decision must be confirmed;
- the UCI appeal must be dismissed;

- the UCI must be ordered to pay costs, in accordance with article 282 of the ADR.

B. THE HEARING

13. The hearing took place on October 11, 2006, at the CAS Head office in Lausanne (The *Hearing*), in the presence of the three members of the Court of Arbitration (the *Panel*) and of the attorneys for the parties.

14. The Panel heard the following witnesses and experts:

- For the UCI:

- Doctor Martial Saugy, director of the Swiss antidoping laboratory (*Dr. Saugy*);
- Professor Jacques de Cœuriz, LNDD director (*Prof. De Cœuriz*).

- For Mr. Landaluce:

- Dr. Douwe de Boer, a researcher at the Clinical Chemistry Laboratory at the Maastricht University Hospital and former director of the Doping Department of the Lisbon Analytical and Doping Laboratory (*Dr. de Boer*);
- Dr. Detlef Thieme, a forensic toxicologist expert at the forensic toxicology department of the Munich Forensic Medicine Institute (*Dr. Thieme*);
- Mr. Ignacio Colomer Hernández, a Law Professor at the Carlos III University in Madrid (*Prof. Colomer*), by videoconference.

C. ON MR. LANDALUCE ENTERING EXHIBITS 43 AND 43 BIS AND ON HEARING MR. MIGUEL MADARIAGA

15. On September 22, 2006, 14 days after filing his response, Mr. Landaluce submitted supplementary exhibits 43 and 43bis.

16. Since the UCI was opposed to said exhibits 43 and 43bis being entered, Mr. Landaluce indicated, by fax dated 2 October 2006, that he wanted Mr. Miguel Madariaga (*Mr. Madariaga*) to testify as a witness.

17. By fax dated 4 October 2006, the UCI opposed the testimony of Mr. Madariaga, since Mr. Landaluce's response did not mention this witness's testimony at all.

18. According to article R56 of the Code of Sports-related Arbitration:

"Unless the parties agree otherwise or the President of the Panel orders otherwise on the basis of exceptional circumstances, the parties shall not be authorized to supplement their argument,

nor to produce new exhibits, nor to specify further evidence on which they intend to rely after the submission of the grounds for the appeal and of the answer."

19. During the hearing, the UCI indicated that it would agree to let Mr. Madariaga testify only if it could add to the debate a new piece of evidence received the day before. The Panel President asked that the evidence be forwarded to the RFEC and to Mr. Landaluce, to make them aware of it. Upon seeing the evidence, Mr. Landaluce dropped his request for Mr. Madariaga to testify.
20. In the absence of "*exceptional circumstances*" in the sense of article R56 of the Code of Sports-related Arbitration, the Panel President decided not to admit the new evidence and not to hear the witness.

III. ON THE COMPETENCE OF THE CAS

21. The organization of professional competitions assumes a clear distribution of competence between national and international authorities, on the one hand, and between the public and private sector, on the other hand.
22. There are domains, such as narcotics trafficking, in which the state requires, in the name of national sovereignty, control over any reprehensible behavior within the country, and refuses to let private associations take charge, including via a delegation of public service.
23. In contrast, discipline in the sports domain, which results in disqualifying the athlete or prohibiting the athlete from participating in future competitions, essentially reflects the will to eliminate unfair competition. This is why it is legitimately entrusted to sports authorities.
24. This is even truer when it comes to the exercise of sport at the international level, which cannot obey a multiplicity of potentially contradictory national rules.
25. To trust national laws alone with the management of international competitions would lead to an incoherent and non-egalitarian system, with the added risk that national authorities would sooner or later try to legislate as leniently as possible.
26. To alleviate such inconveniences, it suffices to ensure that the same sport discipline, when it takes place in an international context, is subject to the same rules for all competitors.
27. Mr. Landaluce tried to cause the Panel to believe that the legal situation in Spain would dictate a different solution. He claimed that the CAS is not competent to hear the UCI appeal against a CNCDD decision and indicates that only the Spanish Committee for Sports Discipline of the Superior Council of Spanish Sports (*SCSD*) is competent.
28. To support his claim, Mr. Landaluce invoked a certain number of texts which are reproduced below:

- Article 6 of the UCI Constitution:

- "1. As members, the federations shall comply with the Constitution and Regulations of the UCI, as well as with all decisions taken in accordance therewith. Likewise, they shall have the Constitution, Regulations and decisions of the UCI complied with by all persons concerned.*
- 2. The Regulations of the UCI shall be incorporated in the corresponding regulations of the federations.*
- 3. The constitution and regulations of the federations shall not run counter to the Constitution and Regulations of the UCI. In case of divergence, only the Constitution and Regulations of the UCI shall apply. The constitution and the regulations of the federations must contain an express clause that in case of divergence with the Constitution and Regulations of the UCI, only the latter shall apply.*
- 4. The foregoing paragraphs shall be applied in observance of the mandatory legal provisions in force in the country of the federation concerned."*

- Article 35 of the Royal Decree 1835/1991 of 20 December 1991 relative to Spanish sports federations:

"The following questions cannot be subject to conciliation or arbitration: [...]

b) Those relative to the control of substances and methods prohibited in sport and the safety of the practice of sport ...]."

- Article 90.2 of the RFEC Constitution in its version following the decision of 11 October 2004:

"The following questions cannot be subject to conciliation: [...]

b) Those relative to the control of substances and methods prohibited in sport and the safety of the practice of sport ...]."

- Section 24 of the Spanish Constitution of 28 December 1978:

"1. All persons have the right to obtain effective protection from the judges and the courts in the exercise of their rights and legitimate interests, and in no case may there be a lack of defense.

2. Likewise, all have the right to the ordinary judge predetermined by law; to defense and assistance by a lawyer; to be informed of the charges brought against them; to a public trial without undue delays and with full guarantees; to the use of evidence appropriate to their defense; not to make self-incriminating statements; not to plead themselves guilty; and to be presumed innocent."

- Article 91.1 of the RFEC Constitution in its version following the decision of 11 October 2004:

"A commitment to undergo the conciliation proceedings will be required from the parties in the litigation. To this end, a written arbitration agreement must include an agreement that the arbitration award shall be final, and the obligation to accept the decision of one or more arbitrators, and the procedural rules."

- Article 84.1 of the Law 10/1990 of 15 October 1990 relative to Sport:

"The Spanish Committee for Sport Discipline is the national organ organically appointed to the Superior Council for Sports which, acting independently from the latter, resolves in final appeal, by administrative procedure, disputes on sports disciplinary questions that fall under its jurisdiction."

- Article 10 of the RFEC Constitution:

"1. Under the coordination and guardianship of the Superior Council for Sports, the RFEC will exercise the following public functions of administrative nature: [...]"

f) Exercise sports disciplinary authority, according to the terms established by the Law on Sport, its particular provisions for development and its statutes and regulations."

- Article 59 of the Royal Decree 1591/1992 of 23 December 1993:

"The competence of the Spanish Committee for Sports Discipline extends to the following:

a. taking notice of and making decisions on appeals concerning the acts of sports entities with disciplinary authority when the remedies offered by sport procedures have been exhausted, according to the distribution of competence established by the Law on Sport and by the present royal decree."

29. Essentially, Mr. Landaluce claimed:

- that arbitration in matters of doping is prohibited in Spain;
- that the Spanish Constitution contains an inalienable right of access to justice and the courts. Mr. Landaluce indicated that he cannot be deprived of this constitutional right of access to justice by being authorized to appeal only before the CAS;
- that he never agreed to CAS arbitration and never signed any arbitration agreement. Mr. Landaluce considers that the mere fact that he holds a RFEC license can in no way constitute acceptance of the competence of the CAS;
- that the RFEC is a public authority exercising public disciplinary functions under the guardianship of a Spanish public entity. Therefore, Mr. Landaluce considers that the RFEC decisions should be subject to an administrative appeal before the CEDS, followed, as needed, by an appeal before the Spanish administrative judge;
- that according to article 6, item 4 of the UCI Constitution, the RFEC must respect Spanish legislation when it applies the UCI standards;

- that articles 242 and 280 of the ADR, which establish the exclusive competence of the CAS to take notice of the appeal against the decisions of national federations, are not applicable, according to article 6.4 of the UCI Constitution, since this exclusive competence would violate Spanish law;

- that the Central Administrative Litigation Court Number 7 of Madrid judgment of 8 June 2006 rendered in a Santiago Perez Fernandez case recognizes the possibility of appeal against the decisions of the CNCDD in a hierarchical sequence: before the SCSD followed, as needed, by an appeal before the Spanish administrative judge.

30. The UCI considers the CAS competent and the ADR applicable, on the basis of the following texts:

- Article 1.1.001 of the UCI Rules:

"The licence is an identity document confirming that its holder undertakes to respect the constitution and regulations and which authorise [sic] him to participate in cycling events."

- Article 1.1.004 of the UCI Rules:

"Anyone requesting a licence thereby undertakes to respect the constitution and regulations of the UCI, the UCI continental confederations and the UCI member Federations, as well as to participate in cycling events in a sporting and fair manner. He shall undertake, in particular, to respect the obligations referred to in article 1.1.023."

As from the time of application for a licence and provided that the licence is issued, the applicant is responsible for any breach of the regulations that he commits and is subject to the jurisdiction of the disciplinary bodies.

Licence holders remain subject to the jurisdiction of the relevant disciplinary bodies for acts committed while applying for or while holding a licence, even if proceedings are started or continue after they cease to hold a licence."

- Article 1.1.023 of the UCI Rules:

*"[...] 2. I hereby undertake to respect the constitution and regulations of the International Cycling Union, its continental confederations and its national federations.
I declare that I have read or have had the opportunity to become acquainted with the aforesaid constitution and regulations.
I shall participate in cycling competitions or events in a sporting and fair manner.
I shall submit to disciplinary measures taken against me and shall take any appeals and litigation before the authorities provided for in the regulations. I accept the Court of Arbitration for Sport (CAS) as the sole competent body for appeals in such cases and under the conditions set out in the regulations."*

I accept that the CAS shall be the court of last instance and that its decisions shall be definitive and without right of appeal. With those reservations, I shall submit any litigation with the UCI solely to the courts within whose jurisdiction the head offices of the UCI lie.

3. I agree to submit to and be bound by the UCI antidoping regulations, the clauses of the World Antidoping Code and its international Standards to which the UCI antidoping regulations refer and to the antidoping regulations of other competent bodies as per the regulations of the UCI and the World Antidoping Code provided that they comply with that Code.[...]"

- Article 5 of the UCI Rules Preliminary provisions:

"Participation in a cycling race, in whatever capacity, shall imply acceptance of all provisions of the Regulations applying thereto."

31. The UCI indicated that the review of the above articles together essentially means that:

- riders who hold licenses from UCI member federations are subject to the ADR;
- any person who applies for a license undertakes to respect the ADR and especially to be subjected to doping control tests and to the competence of the CAS as the court of last instance.

32. To support its claim, the UCI cited the fact that the decision of the CNCDD dated 5 May 2006, which is being appealed, was rendered by applying the ADR.

33. In addition, during the hearing, the UCI claimed that:

- the CAS should assess competence and arbitrability in disputes according to the Swiss federal law on private international legislation (**LDIP**) under whose terms (i) doping can be subject to arbitration, and (ii) arbitration clauses by reference are valid (article 178 of the LDIP);
- according to the CAS jurisprudence, the existence of a national law does not preclude the application of the regulations of the international federation (TAS 2005/A/872, arbitral award dated 30 January 2006, UCI /Federico Muñoz Fernandez and Federación Colombiana de Ciclismo);
- the Spanish legislation invoked by Mr. Landaluce would be intended to apply only in Spain, as shown in the following:
 - (i) article 1.3° of Royal Decree 1835/1991 dated 20 December 1991 on Spanish sports federations, according to which the scope of competence of Spanish sports federations would be limited to within the country;
 - (ii) the preamble of Law 10/1990 dated 5 October 1990 on sport, according to which the object of the law would be to define the legal framework applicable to the practice of sport within Spain.
- article 7 of the Royal Decree 255/1996 dated 16 February 1996, according to which the sanctions imposed in application of the antidoping rules of any federation, be it national,

international or autonomous, have effects in all of Spain, shows that sanctions can be imposed by federations other than RFEC and have effects within Spain;

- to admit, as the sole appeal procedure against CNCDD decisions, an appeal before the SCSD would be equivalent to depriving the UCI of any right of oversight on the decisions arising from the national federation, to the extent that the UCI would not be able to appeal within Spanish jurisdiction.

34. In its writings and during the hearing, the RFEC indicated that it was won over to the UCI's interpretation, with regard to the competence of the CAS. In addition, the RFEC indicated that it was a private entity "*wearing two hats*" exercising, on a case by case basis, missions of a public or private nature. The RFEC stated that in the context of international competitions, such as the case in point, it exercises a mission of a private nature.

35. During the hearing, Prof. Colomer claimed the following:

- the granting of a license by the RFEC constitutes an administrative act;
- the power of sanction of the RFEC is applied in the context of the exercise of a public function;
- the CAS is not competent because the sanction imposed by the CNCDD is an administrative act;
- the fact that arbitration is prohibited in matters of doping is imperative for the parties, with no possible exception;
- the Spanish Constitution prevails over any international treaty;
- any decree contrary to the Spanish Constitution is unconstitutional;
- article 7 of Royal Decree 255/1996 dated 16 February 1006 is unconstitutional.

36. The Panel, aware of the fact that the present dispute illustrates the tension that can exist between an athlete's national law and the regulations of the international federation to which he is subjected, recalls that the UCI is the association of national cycling federations, and that the purposes of the UCI are to direct, develop, regulate, control and discipline international cycling, particularly in matters of doping. As for Mr. Landaluce, he is a professional cyclist in the elite category, holder of a license under the terms of which he accepted to be subject to the UCI regulations.

37. Mr. Landaluce's application for a license certainly shows that the model used by the RFEC to apply for a license does not conform to the UCI Rules. However, articles 2 and 3 of the application for a license signed by Mr. Landaluce indicates that its holder commits himself to respect the current Spanish legislation, the Constitution of the UCI and of its confederations as well, and that he agrees to be subject to doping control tests in the conditions set out in article 1.1.023 of the UCI Rules as well as to the competence of the CAS which is explicitly

set out. The RFEC did indicate, during the Hearing, that the fact of applying for a RFEC license is in itself equivalent to accepting the competence of the CAS, even in the absence of an explicit clause to this effect. The Panel considers that by requesting a license from the RFEC, Mr. Landaluce subjected himself to the competence of the CAS, as well as to the UCI Rules.

38. Some might think that it would have been preferable for the application for a license to mention specifically that the parties agreed to CAS arbitration. In this regard, the Panel emphasizes that elite riders are not lay persons, such as consumers might be, but informed athletes undoubtedly aware of the existence of the CAS and of its competence in matters of doping. Therefore, the competence of the CAS as set out in article 1.1.023 of the Cycling Sport Rules can in no way be a surprise to a person who applies for a license. To require that, in addition to the elements listed in paragraph 37, a license application must mention explicitly the competence of the CAS in order for the CAS to be competent, would be an excessive formality and a demand not required by the jurisprudence of the Swiss federal court.
39. Mr. Landaluce's argument that article 6 paragraph 4 of the UCI Constitution orders the RFEC to apply Spanish law and forbid CAS arbitration doesn't do much to convince the Panel. In fact, article 6 of the UCI Constitution is aimed only at national federations. The first three paragraphs of this article require these federations to harmonize their rules by reiterating the UCI rules. The fourth paragraph of article 6 of the UCI Constitution simply indicates that this harmonization must take into account potential requirements applicable in the federation's country. Consequently, article 6 paragraph 4 of the UCI Constitution explicitly refers to the three preceding paragraphs and cannot be taken out of context. Thus Article 6 of the UCI Constitution addresses only national federations, to indicate to them the procedure to follow to harmonize their rules, and cannot be invoked by a cyclist in order to escape the application of the UCI Rules, and by doing so, the competence of the CAS.
40. After all, no provision of Spanish law excludes arbitration. This is certainly not the case with article 24 of the Spanish Constitution. To be convinced, it suffices to compare the scope of this text to that of article 6 paragraph 1 of the European Convention on Human Rights (*ECHR*) which states that "*everyone is entitled to a fair and public hearing [...] by an independent and impartial tribunal established by law.*" The European Convention on Human Rights decided in the case of X./RFA dated 5 March 1962, that the insertion of a compromissory clause in a contract was equivalent to renouncing the benefit of the ECHR and that no stipulation of the ECHR forbade such a renunciation. Unless otherwise demonstrated, article 24 of the Spanish Constitution, exactly like article 6 paragraph 1 of the ECHR, in no way forbids arbitration. In fact, the competence of the CAS does not deprive Mr. Landaluce of his right to have access to justice as set out by the Spanish Constitution, since arbitration is a form of exercise of this right in an international matter.
41. Finally, the Panel considers that the Spanish provisions invoked by Mr. Landaluce are intended to apply only in the context of national cycling competitions, and cannot be an obstacle to applying the UCI Rules or the competence of the CAS. To decide otherwise would lead to a veritable race for the most lenient national legislation.

42. Indeed, international sports federations such as the UCI must have a right to oversee the decisions of national federations in matters of doping. This right of oversight of the UCI's, which manifests itself by the ability to appeal the decisions of national federations before the CAS, is there to reduce the risk that an international competition would be biased by a national federation that would refrain from sanctioning its members.
43. Therefore, Mr. Landaluce's argument that the sole recourse against the decisions of the CNCDD would be an appeal before the SCSD cannot win the support of the Panel.
44. Likewise, Mr. Landaluce's argument contesting the competence of the CAS because anti-doping rules would be trusted to a public authority is flawed. In fact, a review of CAS jurisprudence indicates that the power of international federations such as the UCI is exercised even when doping control and sanctions are trusted, as in the case in point, to a public authority.
45. The power of international federations is there to eliminate unfair competition and any hunt for the most lenient legislation. It tends to subject all athletes to equal treatment, by seeing to it that certain national federations won't remain passive in view of offenses committed by their country's athletes. Thus, in a doping case involving swimmers in 1997, the CAS recognized as an imperative necessity for international federations to be able to review the decisions of national federations in doping cases. This is to prevent the risk that international competitions would be biased, because a national federation might be tempted to pronounce sanctions that would be too lenient (TAS 96/156, arbitral award dated 10 October 1997, F. / FINA).
46. Later on, another CAS panel had the opportunity to extend this jurisprudence to sanctions pronounced by national public authorities, in B. v. International Judo Federation on 17 March 1999 (TAS 98/214, arbitral award dated 17 March 1999, B./Fédération Internationale de Judo). This was the case of a positive doping control test sanctioned by a suspension imposed by an order of the French Minister of Sports. Indeed, a 1989 French law gave said minister the power to substitute his decision to any punitive measure taken by national sports federations. In the case in point, the French Federation had imposed a two-year suspension, of which one year was stayed, and the ministry's order substituted a simple one-year suspension. However, the latter sanction was not in conformity with the rules of the International Judo Federation (*IJF*), which brought the case before the CAS. The latter ordered a 15-month suspension.
47. In that arbitral award, the arbitrators evoked the problem that is at the heart of the present dispute:
- "The panel's opinion is that the latitude granted by this jurisprudence to international federations must be extended to the cases in which the doping control procedure and doping sanction are not handled by a national federation, according to sport rules, but by a public authority applying a national law, as in the case in point, or, in relevant cases, based on an international convention."*

The power to extend, at the international level, national decisions relative to doping, no matter what authority rendered them, is justified not only to prevent the risk that certain federations or government entities would engage in unfair competition of the unhealthiest sort, by failing to sanction their athletes as rigorously and severely as other federations and/or their international federations, but also to reach the goal that each international federation must try to achieve, which is to make all participants of the same sport respect an equal and consistent treatment."

48. Therefore, the provisions of the Spanish law that are referred to do not allow to conclude to the lack of competence of the CAS. This is what the CAS determined on several occasions, particularly in cycling in the Muñoz case (TAS 2005/A/872, arbitral award dated 30 January 2006, UCI /Federico Muñoz Fernandez and Federación Colombiana de Ciclismo):

"The panel is prepared to accept that as a matter of Colombian Law it was possible for Mr. Muñoz to appeal to the General Disciplinary Committee of the Colombian National Olympic Committee. However, to do so was a breach of his contract with the UCI. At best, the decision of the General Disciplinary Committee could only have an effect within Colombia. It would not entitle Mr. Muñoz to participate in cycle races organized under the auspices of the UCI, or to avoid the UCI's disciplinary code." [in English in the original document]

49. The authority of States and that of international sport are not in competition; on the contrary, their roles are complementary. The state authority is limited to controlling the behavior of persons subject to its jurisdiction, whereas the international federation manages the competitions that fall under its jurisdiction. One and the same behavior can lead to a penal sanction in a given place, without leading to the cyclist being sanctioned at the international level. Likewise, a behavior can lead to no penal sanctions while it can generate an exclusion from sports contests because it damages the fairness of competition.

50. This complementarity between state and international sport authorities can take on a particular form when a public authority substitutes itself to the national federation to decide on sanctions—as was the case in the above IJF case, or in the case in point. National sovereignty, as expressed when a sport disciplinary measure is taken by a national authority, applies in principle only within the country. However, the national decision can be replaced by a decision of the international authority—the CAS—in order to ensure the necessary legal uniformity. It is certainly theoretically conceivable that the State would impose its national decisions in international competitions taking place within the country, disregarding the international authority. Such a behavior, however, would go against all the efforts made in the fight against doping at the international level, and could lead to excluding that State from organizing international competitions. It would be surprising for a State to want to put itself in such a position, and there is nothing in the texts invoked in the present case that would suggest that Spain would have adopted this position. Quite the contrary, the preamble of the Royal Decree 255/1996 dated 16 February 1996 clearly reflects the fact that Spain is concerned with whether its standards conform to international standards:

"In accordance with article 76.1.d) of the Law on Sport and in accordance with the criteria of international sports standards, the present Royal Decree identifies the behaviors that constitute doping infractions and establishes the relevant sanctions [...]."

51. Far from supporting Mr. Landaluce's claim, the Royal Decree 255/1996 dated 16 February 1996 that he invokes only confirms the reasoning developed above. Indeed, article 7 of this Decree explicitly recognizes that the sanctions imposed by an international federation have effects on all of Spain. The contrary would have been surprising, to say the least.
52. In view of the above, the Panel concludes that it is competent in the present litigation, in accordance with articles 280 to 291 of the ADR.
53. This is in fact not contested by the RFEC which has, during the entire proceedings, recognized the competence of the CAS and the applicability of the ADR, which were explicitly referred to in the CNCDD decision of 5 May 2006, which is the subject of the present appeal.

IV. ADMISSIBILITY

54. Article 285 of the ADR states that the UCI appeal brief must be filed with the CAS within a month after receiving the complete dossier of the national federation hearing. However, when the UCI has not requested the dossier within 15 days after receipt of the decision, as per article 247 of the ADR, the deadline for appeal is one month from receipt of the complete decision.
55. In the case in point, the UCI received the CNCDD decision on 9 May 2006 and requested the complete dossier on 11 May 2006. The complete dossier was received by the UCI on 26 May 2006. The appeal brief was sent on 26 June 2006, by the deadline.

V. MERITS

A. GENERAL PRINCIPLES

56. According to article 16 of the ADR:

"The UCI and its National Federations shall have the burden of establishing that an anti-doping rule violation has occurred. The standard of proof shall be whether the UCI or its National Federation has established an anti-doping rule violation to the comfortable satisfaction of the hearing body bearing in mind the seriousness of the allegation which is made. This standard of proof in all cases is greater than a mere balance of probability but less than proof beyond a reasonable doubt. Where these Anti-Doping Rules place the burden of proof upon the Rider or other Person alleged to have committed an anti-doping rule violation to rebut a presumption or establish specified facts or circumstances, the standard of proof shall be by a balance of probability."

57. On the methods of establishing facts and presumptions, article 18 of the ADR sets out the following:

"WADA-accredited laboratories or as otherwise approved by WADA are presumed to have conducted Sample analysis and custodial procedures in accordance with the International Standards for laboratory analysis. The Rider may rebut this presumption by establishing that a departure from the International Standard occurred.

If the Rider rebuts the preceding presumption by showing that a departure from the International Standard occurred, then the UCI or the National Federation shall have the burden to establish that such departure did not cause the Adverse Analytical Finding."

In other words, there is a refragable presumption that WADA-accredited laboratories conduct analyses in accordance with prevailing and acceptable standards of scientific practice, in accordance with the International Standard for Laboratories (ISL). In the event that the rider demonstrates a departure from the ISL, it is then the UCI's burden to demonstrate that this departure is not at the origin of the adverse analytical finding. Therefore these rules must be applied to determine whether Mr. Landaluce has established that there were departures, and if so, whether the UCI can demonstrate that the departure cannot have caused the adverse finding.

58. According to article 15.1 of the ADR:

"The following constitute anti-doping rule violations:

1. The presence of a Prohibited Substance or its Metabolites or Markers in a Rider's bodily Specimen.

1.1 It is each Rider's personal duty to ensure that no Prohibited Substance enters his body. Riders are responsible for any Prohibited Substance or its Metabolites or Markers found to be present in their bodily Specimens. Accordingly, it is not necessary that intent, fault, negligence or knowing Use on the Rider's part be demonstrated in order to establish an anti-doping violation under article 15.1."

59. The substance testosterone is an endogenous androgenic anabolic steroid, a prohibited substance listed in the 2005 Prohibited List in class S1.1.b. The prohibited lists states the following:

"Where a Prohibited Substance (as listed above) is capable of being produced by the body naturally, a Sample will be deemed to contain such Prohibited Substance where the concentration of the Prohibited Substance or its metabolites or markers and/or any other relevant ratio(s) in the Athlete's Sample so deviates from the range of values normally found in humans that it is unlikely to be consistent with normal endogenous production. A Sample shall not be deemed to contain a Prohibited Substance in any such case where the Athlete proves by evidence that the concentration of the Prohibited Substance or its metabolites or markers and/or any other relevant ratio(s) in the Athlete's Sample is attributable to a physiological or pathological condition. In all cases, and at any concentration, the laboratory will report an Adverse Analytical Finding if, based on any reliable analytical method, it can show that the Prohibited Substance is of exogenous origin.

If the laboratory result is not conclusive and no concentration as referred to in the above paragraph is found, the relevant Anti-Doping Organization shall conduct a further investigation if there are serious indications, such as a comparison to reference steroid profiles, for a possible Use of a Prohibited Substance.

If the laboratory has reported the presence of a T/E ratio greater than four (4) to one (1) in the urine, further investigation is obligatory in order to determine whether the ratio is due to a physiological or pathological condition, except if the laboratory reports an Adverse Analytical Finding based on any reliable analytical method, showing that the Prohibited Substance is of exogenous origin."

B. APPLICATION OF THE GENERAL PRINCIPLES TO THE PARTICULAR CASE

60. In this particular case, the LNDD used, as an analysis method, GC/C/IRMS, analysis by isotopic ratio mass spectrometry. The LNDD analytical report states the presence of exogenous testosterone. The A sample had a T/E ratio of 9.2, greater than the limit of 4 established by WADA. The LNDD B sample analytical report confirmed the A sample analysis result.
61. Since the LNDD conducted analyses whose results are positive, it is Mr. Landaluce's burden to demonstrate that there was a departure from the ISL in order to reverse the presumption that the analyses were conducted in accordance with prevailing and acceptable standards of scientific practice. As justifiably recalled by UCI during the hearing, Mr. Landaluce may not simply indicate that *"something could potentially be wrong"* [quote in English in the original document], but must indeed prove that there was a violation of the ISL.
62. To do so, Mr. Landaluce contested the validity of the LNDD analyses, invoking failures and irregularities during the process. He claimed that there irregularities *"invalidate"* the analysis results and indicated that *"these results will never be considered to prove that a fault was committed that can be sanctioned."*
63. To this end, Mr. Landaluce emphasized that he relied on the observations of Dr. de Boer and of Mrs. Luisa Lurueña Sánchez, a pharmacist and expert in the analysis and quality control of medicines and drugs.
64. Mrs. Luisa Lurueña Sánchez noted the following:
- "The documentation provided is insufficient to demonstrate the abuse of testosterone or a precursor.*
Given the bad, weak and inconsistent analytical documentation shown to demonstrate the abuse of testosterone or analogs in this sample, the athlete's innocence can be defended."
65. In support of his claims, Mr. Landaluce also provided numerous reports by Dr. de Boer who claimed that several irregularities were committed by the LNDD and emphasized that the ISL was not met. In his report dated 12 March 2006, Dr. de Boer stated the following in particular:

"The WADA laboratory in Châtenay-Malabry made no mistake with regard to the chemical analysis, but it did not meet, in several ways, the WADA international standards for analytical Laboratories (ISL, International Standards for Laboratories). Therefore, several nonconformities occurred, which, among other things, resulted in the "A" and "B" sample analysis being far from complete."

66. Mr. Landaluce invoked six departures from the ISL allegedly committed by the laboratory. Four of the alleged departures have to do with scientific interpretation by the laboratory, whereas the other two have to do with the proceedings initiated by him. The Panel must examine the alleged irregularities invoked by Mr. Landaluce to determine whether the LNDD met the ISL.

1. Alleged failures concerning scientific interpretation by the LNDD

a) The argument that the documentation does not allow another analyst to evaluate the analyses and interpret data (point 5.2.6.1 of the ISL)

67. According to point 5.2.6.1 of the ISL:

"The Laboratory must have documented procedures to ensure that it maintains a coordinated record related to each Sample analyzed. In the case of an Adverse Analytical Finding, the record must include the data necessary to support the conclusions reported (as set forth in the Technical Document, Laboratory Documentation Packages) In general, the record should be such that in the absence of the analyst, another competent analyst could evaluate what tests had been performed and interpret the data."

68. Dr. de Boer declared that he could not evaluate different aspects of the analyses because of a lack of information and indicated that the A and B sample reports did not contain information allowing the identification of the steroids in question.

69. As for Dr. Saugy, he indicated that the information allowed the identification of the steroids in question. Indeed, in his declaration dated 14 July 2006, Dr. Saugy indicated that *"after careful and reasonable review of the documentation packages, I have determined that all the elements allowing an evaluation of the dossier are included in it."*

70. The Panel notes that there is a disagreement between the experts. Whereas Dr. Saugy found that the documentation made it possible to identify the steroids in question, Dr. de Boer considered that the elements at his disposal were insufficient. The arbitrators consider that Dr. Saugy's testimony is plausible and that Dr. de Boer's demonstration does not invalidate Dr. Saugy's analysis.

71. Therefore, the Panel's opinion is that Mr. Landaluce has not demonstrated a violation of point 5.2.6.1 of the ISL and that he has not succeeded in reversing the presumption according to which the LNDD analysis was done in accordance with prevailing and acceptable standards of scientific practice, as per point 5.2.6.1 of the ISL.

b) the argument that the substance found is a threshold substance and that the uncertainty of 0.8 adopted by the LNDD would be insufficient (point 5.4.4.1.3 of the ISL)

72. According to point 5.4.4.1.3 of the ISL:

"For both Non-threshold and Threshold Substances, the Laboratory will be required to meet a Minimum Required Performance Limit for detection, identification, and demonstration that a substance exceeds the threshold (if required)."

73. Dr. de Boer's opinion was that the uncertainty value of 0.8 presented by the LNDD was insufficient and should be 1.35. In addition, he indicated that the fact that an uncertainty index was given shows that the LNDD considered the substance to be a threshold substance to the extent that uncertainty is taken into account only for threshold substances. Dr. de Boer concluded that there was a departure from point 5.4.4.1.3 of the ISL.

74. As for Prof. de Ceaurriz and Dr. Saugy, their opinion was that this value, used by many laboratories, is in compliance with point 5.4.4.1.3. of the ISL. In his declaration dated 14 July 2006, Dr. Saugy did not consider it to be a measurement of a threshold substance, but a confirmation method based on quantitative measurements, whose goal is to demonstrate the qualitative origin of the product ingested.

75. The UCI argued that it is not a threshold substance to the extent that the mere presence of exogenous testosterone suffices. The isotopic analysis aims solely to demonstrate the exogenous nature of the product, without any concern for its quantity.

76. To support its allegation, the UCI referred to point 5.4.4.3 of the ISL which indicates the following:

"In most cases an identification of a Prohibited Substance, its Metabolite(s) or Marker(s), is sufficient to report an Adverse Analytical Finding. Thus, quantitative uncertainty as defined in ISO/IEC 17025 does not apply. In the identification of a compound by GC/MS or HPLC/MS, there are qualitative measures that substantially decrease the uncertainty of identification.

In the case of a Threshold Substance, uncertainty in both the identification and the finding that the substance is present in an amount greater than the threshold concentration must be addressed."

77. In his declaration dated 14 July 2006, Dr. Saugy noted that the WADA Technical Document TD2004EAAS indicates that the result demonstrates the administration of an exogenous steroid when the metabolite value differs significantly by 3% or more from that of the endogenous reference chosen. Dr. Saugy declared that in the case in point, the analysis revealed, for both the A and B samples, a difference significantly greater than 3%, namely 4.85% for the A sample and 4.76% for the B sample, between the endogenous reference and the metabolite found in the testosterone.

78. The Panel notes that there is no precise method in this matter and that the experts present at the Hearing did not reach agreement on an uncertainty value to take into account for isotopic analysis.

79. The Panel's opinion is that Mr. Landaluce has not reversed the presumption that the LNDD conducted its work in accordance with prevailing and acceptable standards of scientific practice. Indeed, the experts cited by Mr. Landaluce, although they demonstrated their disagreement with the calculation method adopted by the LNDD, have not reversed the presumption that the analyses were in compliance with point 5.4.4.1.3 of the ISL. Therefore, the Panel finds that Mr. Landaluce has not proved that there was a departure from point 5.4.4.1.3 of the ISL.

c) The argument that the requested information was not provided to the cyclist or to his expert (point 5.4.7.3 of the ISL and of technical documents TD2004EAAS and TD2003IDCR)

80. Mr. Landaluce argued that he did not receive the information requested several times, by him as well as by Dr. de Boer, which would have made it possible to evaluate the analysis done by the LNDD.

81. The UCI indicated that RFEC correspondence dated 22 November 2005 shows that Mr. Landaluce received the totality of the documents relative to the A and B sample analyses. The UCI also argued that correspondence containing the requested information was sent directly by the LNDD to the RFEC. This correspondence was received safely by the RFEC which stamped it, despite the fact that the CNCDD decision indicates that no response from the LNDD was received.

82. In addition, Dr. de Boer indicated that he received on 29 August 2006 a document containing the evaluation of the retention times for the identification of the steroids in question in the B sample analysis. However, he noted that no information was communicated to him regarding mass spectral and identification data on the steroids involved in the A sample analysis. Therefore, Dr. de Boer's opinion was that there was a departure from point 5.4.7.3 of the ISL and technical documents TD2004EAAS and TD2003IDCR.

83. The Panel notes once more a divergence of opinion between the experts present at the Hearing. Indeed, Dr. Saugy indicated that the information necessary for the identification of these products were provided by the LNDD. This analysis by an expert who enjoys non-negligible experience in this field is quite plausible. Therefore, the Panel considers that Mr. Landaluce has not reversed the presumption that the LNDD complied with point 5.4.7.3 of the ISL and technical documents TD2004EAAS and TD2003IDCR.

d) The arguments that the values do not significantly differ from 3 delta units or more and that three aliquots should have been analyzed (technical document TD2004EAAS)

84. According to the technical document TD2004EAAS:

"The results will be reported as consistent with the administration of a steroid when the 13C/12C value measured for the metabolite(s) differs significantly i.e. by 3 delta units or more from that of the urinary reference steroid chosen. In some Samples, the measure of the 13C/12C value of the urinary reference steroid(s) may not be possible due to their low concentration. The results of such analyses will be reported as "inconclusive" unless the ratio measured for the metabolite(s) is below -28‰ based on non-derivatised steroid."

85. According to Dr. de Boer, this technical document requires that the values differ significantly from three delta units or more. In addition, since this is a threshold value, the ISL requires that three aliquots be analyzed, or as in the case in point, when only one aliquot is available, that it be analyzed three times.
86. As for Prof. de Ceaurriz and Dr. Saugy, their opinion was that triplicate sample measurements are not necessary and that such a requirement, which does not even exist, would on the contrary be problematic when the necessary urine volume is not available. They indicate that this is not a threshold analysis requiring the analysis of several samples.
87. The Panel notes once more that there is a difference in interpretation between the experts and considers that Mr. Landaluce has not reversed the presumption that the LNDD complied with technical document TD2004EAAS.

2. Alleged failures concerning the procedure followed by the LNDD

a) the argument that deadlines were not met (points 5.2.4.3.2.1 and 5.2.6.5 of the ISL)

88. Dr. de Boer claimed that the LNDD did not meet the deadlines described by points 5.2.4.3.2.1 and 5.2.6.5 of the ISL.

89. According to point 5.2.4.3.2.1 of the ISL:

"In those cases where confirmation of a Prohibited Substance, Metabolite(s) of a Prohibited Substance, or Marker(s) of the Use of a Prohibited Substance or Method is requested in the "B" Sample, the "B" Sample analysis should occur as soon as possible and should be completed within thirty (30) days of notification of an "A" Sample Adverse Analytical Finding."

90. According to point 5.2.6.5 of the ISL:

"Reporting of the "A" Sample results should occur within ten (10) working days of receipt of the Sample. [...] The reporting time may be modified by agreement between the Laboratory and the Testing Authority."

91. Dr. de Boer emphasized that the A sample results, which should have been reported within 10 working days of 14 June 2005, the date of receipt of said sample by the LNDD, were reported only 23 days later, i.e., 17 July 2005. Dr. de Boer also indicated that the B sample analysis was not done by the deadline of thirty days after notification of the A sample adverse analytical finding. Indeed, the A sample analysis result notification occurred on 28

July 2005. The LNDD proposed the date of 11 September 2005 for the opening of the B sample, a date which, upon request by Mr. Landaluce, was postponed to 21 September 2005, then rescheduled for 19 October 2005.

92. The UCI recognized that deadlines were not met, invoking *"laboratory overload, the complexity of the analysis and the vacation period."*
93. During the Hearing, the group of experts present declared that the delays had no effect on the analyses results.
94. In accordance with the rules relative to the method of establishing facts and presumptions, the Panel finds that Mr. Landaluce succeeded in reversing the presumption that the LNDD complied with points 5.2.4.3.2.1 and 5.2.6.5 of the ISL by demonstrating the existence of a departure. Such a demonstration, however, has only a purely theoretical effect, since the group of experts present at the Hearing observed that the departure observed could not be at the origin of the positive results of the A and B sample analysis. Therefore, the Panel concludes that Mr. Landaluce's adverse finding does not result from the departures from 5.2.4.3.2.1 and 5.2.6.5 of the ISL.

b) The argument that the analyst who did the analysis of the B sample was also involved in the analysis of the A sample (point 5.2.4.3.2.2 of the ISL)

95. According to point 5.2.4.3.2.2 of the ISL:

"The "B" Sample confirmation must be performed in the same Laboratory as the "A" Sample confirmation. A different analyst must perform the "B" analytical procedure. The same individual(s) that performed the "A" analysis may perform instrumental set up and performance checks and verify results."

96. Mr. Landaluce used as a basis the report of Dr. de Boer to claim that the analyst who did the analysis of the B sample was also involved in the analysis of the A sample, in violation of point 5.2.4.3.2.2 of the ISL.
97. Dr. de Boer indicated that this standard prohibits the same analyst from touching/manipulating both the A and B samples (*"touching the sample must be separate"*). [quote in English in the original document]
98. In this particular case, the report dated 11 June 2005 reveals that the analyst who did the analysis of the B sample did the following tasks in the A analysis: *package* [in English in the original document] at 4 degrees C, redissolve in acetonitrile and transfer to a vial, evaporate, redissolve in hexane and inject in the GC-MS.
99. The Panel President asked Dr. Saugy whether point 5.2.4.3.2.2 of the ISL prohibited the same analyst from touching/manipulating both the A and B samples. Dr. Saugy acquiesced in the following terms: *"I agree that it excludes any manipulation of the sample"*.

100. The Panel President then interrogated Prof. de Ceuriz to know whether the same analyst had touched/manipulated the A and B samples. Prof. de Ceuriz replied:

"Yes. It is clearly indicated. If you want, it is indicated in our chain of [custody]. There is no ambiguity on this. [The analyst] touched the samples in the A and touched the totality of the samples in the B. There is no ambiguity on this."

101. The Panel President then asked whether that constituted a departure from the ISL, to which Prof. de Ceuriz replied:

"Indeed. It is even openly in the laboratory documents. [...] with respect to the standard, that is true, she had contact with the sample".

102. Prof. de Ceuriz indicated that there had been 10% of "overlap between the two persons for workload reasons".

103. The group of experts present at the Hearing thus recognized that the analyst who participated in the two analyses did not limit herself to "performing instrumental set up and performance checks and verify results" and determined that there had been a departure from point 5.2.4.3.2.2 of the ISL.

104. In accordance with the rules relative to the method of establishing facts and presumptions, the Panel determines that Mr. Landaluce succeeds one more time in reversing the presumption according to which the LNDD would have acted in conformity with point 5.2.4.3.2.2 of the ISL, by demonstrating the existence of this departure. The consequences this time are decisive.

105. It was not demonstrated that this was not at the origin of the adverse finding, nor that it was. It was however incumbent upon the UCI, according to article 18 of the UCI Anti-doping Rules, to demonstrate that the departure from the ISL was not at the origin of the adverse finding, but this was not done. The UCI merely indicated in its appeal brief that:

"And even if there had been a departure—quod non—this couldn't have led to the adverse analytical finding, unless it is established that [the analyst] committed an error which caused the adverse analytical finding, quod non."

106. Also during the Hearing, the UCI simply noted:

"As for the departures from the ISL which were brought up, I believe I can conclude that if they took place, they are not significant and are certainly not at the origin of the result."

107. It was indeed for the UCI to demonstrate that the failure to meet point 5.2.4.3.2.2 of the ISL was not at the origin of the adverse finding. To the extent that the UCI did not succeed in doing so, the Panel's only possible conclusion is to exonerate Mr. Landaluce.

108. It is true that a decision pronounced on this question of the double involvement of the same analyst in examining A and B samples, in the case of Wang Lu Na and others versus FINA (TAS 98-208, decision dated 22 December 1998, Wang Lu Na and others versus

FINA) upheld the suspension of four swimmers. However, what that decision brings to the needs of present case is very marginal, for three reasons: Firstly, the applicable regulation is different. Then, the decision expressly indicates (*paragraph 5.22*) that the role played by the analysts in question was not clearly established: the arbitrators declared that they were "*unpersuaded*" by the argument according to which the analysts "*were involved in anything other than the tests of the controls*" as opposed to "*A samples themselves*". [quotes in English in the original document] In contrast, in the case in point, as demonstrated previously, the analysts who manipulated the A and B samples are one and the same. Finally, the swimmers' representatives had, by their signatures, recognized that the procedure described by the FINA regulation had been respected by the laboratory. In the case in point, the UCI did not follow up on presenting evidence that Mr. Landaluce had approved the procedure that was followed. [French grammar questionable]

109. Although aware of the imperatives of costs and organization faced by laboratories, the Panel must watch over the respect of fundamental rules, considering the implications that its decision could have on the reputation, and therefore, the career of the athlete, if a disciplinary sanction were to be pronounced against him.
110. The Panel is well aware that the standard which requires that a different analyst analyze the B sample has been the subject of intense discussions between WADA and laboratory directors. The latter claim that this rule unreasonably complicates laboratory operations, and yet it has not been demonstrated that it brings additional protection to the athletes tested. Indeed it would be unrealistic to require that the same analyst conduct the totality of an analysis from beginning to end. In fact, the analyses for certain substances can last several days during which processes are mechanically carried out. The analysts carry out numerous tasks, shifting from one to the other, so that several analyses can be done simultaneously. If it is conceivable to require of a large laboratory with a staff of 50 to 100 to organize the work so as to exclude from the analysis of the B sample the analyst who analyzed the A—even though this constitutes a non-negligible complication factor which the laboratories would rather be spared—such a requirement would constitute a major complication factor for a laboratory of smaller size.
111. It is virtually impossible to prove a negative fact, in this case that the involvement of the same analyst in both analyses did not affect the result. Therefore certain laboratory directors consider this rule too rigid; in reality, sufficient protection of the athletes is already ensured in that the system of identification of samples by codes ensures that their identity is not known to the analysts.
112. This reasoning, although rational and plausible, fails before the CAS for a very simple reason: the arbitrators do not create the rules, they apply them. This is all the more true because the authors of the antidoping regulation kept the rule which requires another analyst for the analysis of the B sample, even though they had heard the comments of the laboratory directors. The rules can certainly be modified or refined, but such is not the role of the CAS.

119. Articles R64.4 and R64.5 of the Code state the following:

"At the end of the proceedings, the Court Office shall determine the final amount of the cost of arbitration, which shall include the CAS Court Office fee, the administrative costs of the CAS calculated in accordance with the CAS scale, the costs and fees of the arbitrators calculated in accordance with the CAS fee scale, a contribution towards the expenses of the CAS, and the costs of witnesses, experts and interpreters. The final account of the arbitration costs may either be included in the award or communicated separately to the parties."

"The arbitral award shall determine which party shall bear the arbitration costs or in which proportions the parties shall share them. As a general rule, the award shall grant the prevailing party a contribution towards its legal fees and other expenses incurred in connection with the proceedings and, in particular, the costs of witnesses and interpreters. When granting such contribution, the Panel shall take into account the outcome of the proceedings, as well as the conduct and the financial resources of the parties."

120. In this case, the UCI appeal is dismissed and the decision being appealed must be confirmed. This arbitration being at no cost to the parties, since it is a disciplinary case of an international nature, it is necessary to determine whether and to what extent Mr. Landaluce and the RFEC have a right to an indemnity for their attorneys' costs and for the other costs relative to the present arbitration proceedings. After having duly considered the overall circumstances of the case, particularly the large number of futile arguments invoked by the defendants, the Panel considers it equitable to let each party pay the costs incurred in this procedure.

VII. CONFIDENTIALITY

121. According to article R59 paragraph 6 of the Code of Sports-related Arbitration:

"The award, a summary and/or a press release setting forth the results of the proceedings shall be made public by the CAS, unless both parties agree that they should remain confidential."

122. Mr. Landaluce indicated that he wished for the proceedings to remain confidential and that no press release setting forth the proceedings be made public by the CAS.

123. The UCI indicated that the proceedings initiated against Mr. Landaluce were already known to the public, since they have been the subject of a press release by the CAS.

124. During the Hearing, the UCI, Mr. Landaluce and the RFEC did not reach agreement on the confidential or non-confidential character of the present proceedings. Under these circumstances, the CAS will be able, if it deems it appropriate, to make public a summary and/or a press release setting forth the results of the proceedings.

ON THESE GROUNDS

The Court of Arbitration for Sport rules that:

1. The Court of Arbitration for Sport is competent to rule on the appeal submitted by the Union Cycliste Internationale.
2. The appeal submitted on 26 June 2006 by the Union Cycliste Internationale against the decision rendered on 5 May 2006 by the Comité Nacional de Competición y Disciplina Deportiva de la Real Federación Española de Ciclismo is dismissed.
3. The award is rendered at no cost, except for the Court Office Fee of 500 Swiss francs paid at the beginning of the proceedings by the Union Cycliste Internationale, which shall be kept by the CAS.
4. Each party shall bear the costs of its attorneys and other costs associated with the present proceedings.

Lausanne, 19 December 2006.

COURT OF ARBITRATION FOR SPORT

President of the Panel

Jan Paulsson

Olivier Carrard
Arbitrator

José Juan Pintó
Arbitrator

Shaparak Saleh
Ad-hoc clerk

CCA 05293

INTERNATIONAL FEDERATION OF CLINICAL CHEMISTRY

Testing urine for drugs

D. Catlin, D. Cowan, M. Donike, D. Fraisse, H. Oftebro and
S. Rendic

*Departments of Medicine and Pharmacology, University of California, Los Angeles, UCLA Medical Center,
650 Circle Drive South, Los Angeles, CA 90024 (USA)*

Key words: FCC; Urine testing; Doping; Gas chromatography-mass spectrometry; Drug abuse;
Drug testing

I. Introduction

The rising demand for drug testing comes from societies' pressures to stem the spread of substance abuse and to provide greater protection to their members. In response to this demand urinalysis testing programs have been implemented by a wide variety of organizations such as business and industrial employers, the transportation industry, police and fire departments, the military and sports [1–7]. The drugs most commonly tested for are marijuana, cocaine, amphetamines and narcotic analgesics. Some programs also test for benzodiazepines, barbiturates and phencyclidine.

One common denominator is the widely accepted notion that drug testing will identify the primary offenders. The extent to which this objective is achieved is most appropriately addressed by specialists in behavioral medicine and sociology. The scientific basis for urinalysis programs is in the domain of clinical and analytical chemistry.

The analysis of body fluids for drugs takes place in two distinctly different environments: the medical model and the penalty model. In the former and in the context of patient care, the physician requests the test and the patient is both fully cooperative with the sample collectors and highly motivated to provide the ideal

Correspondence to: Don H. Catlin, M.D., Chief, Clinical Pharmacology, Departments of Medicine and Pharmacology, University of California, Los Angeles, UCLA Medical Center, 650 Circle Drive South, Los Angeles, CA 90024, USA

The copyright is vested in IFCC.

sample. In the latter case the request usually comes from a non-medical authority and it may not be in the best interest of the individual to submit a valid sample. In the medical model the information obtained from the test is used to assist the physician in the care and management of the patient. In contrast, in the penalty model the results are used to impose some penalty to the individual. This fundamental difference together with legal imperatives has broad implications for the overall design and implementation of a drug testing program.

Although it may seem counter-intuitive, testing in the penalty model is far more demanding and difficult compared to the medical model. For example, if a physician sends a sample from an unconscious patient to the laboratory for a rapid screen and, 1 h later, the report is positive for opiates by immunoassay, the physician will use this information to tailor treatment to the opiate overdose as opposed to say the barbiturate overdose. It is relatively unimportant to know if the primary drug is morphine or codeine because the clinical implications are very similar. In contrast, if the urine came from a pre-employment screening program, it would be of utmost importance to know if the positive test was due to the consumption of heroin, a dangerous and illicit drug, or codeine, a drug which is widely available and commonly prescribed for pain. In fact since urinary morphine could arise from the ingestion of morphine, heroin, or codeine the complete analysis of this sample requires a specific and quantitative analysis for all metabolites of all three substances. Furthermore this type of analysis virtually requires gas chromatography-mass spectrometry (GC-MS), a technique which is inherently difficult, labor intensive and expensive. Thus the complete analysis of a complex sample is generally beyond the traditional scope of hospital or clinic based clinical chemistry laboratories and serves to emphasize that the clinical chemist who wishes to extend his practice to drug testing must be prepared for a substantial increase in analytical instrumentation and technical training. Further amplification of this point appears in numerous recent publications and reviews [3,4,8].

Additional factors which must be considered in the testing with penalties model include constant scrutiny by the legal community, the activity of regulatory agencies, scrupulous attention to chain of custody both within and without the laboratory, space allocation to provide for specialized functions and security and development of substantial knowledge regarding the pharmacology, pharmacokinetics and metabolism of the substances. In order to accommodate these considerations the traditional laboratory must reorganize administrative procedures, job descriptions, security, laboratory protocols and space allocation.

Urine testing of sportpersons (doping control) differs from that of employees in that the main reason to test is to maintain fair play by prohibiting the use of performance-enhancing drugs, as opposed to protecting safety or productivity on the job by prohibiting street drugs. In marked contrast to testing for the so called 'drugs of abuse', the menu of drugs tested for by sport authorities is both comprehensive and includes several classes of substances [1,9]. Some banned substances are also produced endogenously (e.g. testosterone) and in the case of several substances the ban is based on quantification. Detection of the administration of exogenous testosterone requires special testing techniques [8,10,11].

The current list of substances banned by the International Olympic Committee

consists of six groups [8], i.e. the stimulants where several classes are found such as the psychomotor stimulants (ex: amphetamine), the sympathomimetics, (ex: ephedrine), and the analeptics (ex: strychnine, caffeine). The other groups are narcotics, anabolic steroids, beta-blockers, diuretics and certain peptide hormones. This classification is based on the expected effect of the drugs on the athlete as opposed to their chemical structure and properties.

II. Regulatory Considerations

Legal review can be expected [12], therefore laboratory directors should be familiar with the relevant local, national and international laws. In general such reviews are designed to find fault with any aspect of the system and in particular the authority to test, provisions for individual consent to undergo testing, collection procedures, chain of custody, sample chemistry, instrument maintenance, tuning and calibration records, quality control, the analytical data consisting of chromatograms and spectra, sample storage, qualification of laboratory personnel, confidentiality and appeal process. Laboratories are advised to review carefully the documents which describe the authority to test and the details of the client's protocol. The best defense for an unfavorable legal decision is excellence in analytical chemistry together with a complete set of written procedures covering all aspects of the testing program and documentation showing that all procedures were followed.

Various countries and scientific organizations either have produced or are in the process of preparing guidelines to regulate drug testing laboratories [2,4-6]. These are designed to protect the person tested ('testee') by improving the quality of the work product by emphasis on quality assurance, quality control, proficiency testing and documentation. In some countries the guidelines are incorporated into accreditation or certification programs [5]. Consumers of laboratory services are encouraged to use only those that are certified or accredited.

III. Sample Collection Procedures

Prior to urine collection the testee is offered the opportunity to declare all medications and related substances taken recently. This information becomes part of the documentation, and in the event of a positive test and legal action, it is used by the certifying scientist to evaluate the possibility of analytical interferences or inadvertent use of a banned substance.

It is essential to ensure that the urine tested is authentic urine from the designated individual. Experience has shown that some individuals will attempt to evade submitting authentic urine by techniques such as concealing bladders and tubing under their clothing and even introducing 'clean' urine into their bladder just before the test [13]. To help exclude these measures and other forms of substitution, the testee should remain under the constant supervision of at least one member of the drug testing team from the time of notification to the time of urination. Next the drug testing official must verify the identity of the testee by requesting a passport, driver's license, or otherwise accepted document with photograph. Finally the actual urination should be directly observed by a drug testing team member of the same gender

(cf. Ref. 15). Some organizations go to further extremes and require removal of all clothing. Others do not directly observe urination, but use temperature-sensitive containers [5].

To ensure sample integrity, the urine collection containers should be individually sealed. The testee is given the opportunity to select any container which is used to collect or ship the specimen. No one but the testee should handle the urine in the container and/or the bottles until they are sealed. The testee and officials all sign a declaration that protocol was followed to their satisfaction. The laboratory should not know the identity of the testee, therefore urine samples are forwarded to the laboratory identified only by a number. The organization retains the confidential master code which links each number to an individual's name. To exclude all varieties of sabotage, the collection area should be secure and have restricted access such that no unauthorized person can enter.

Because there are manipulations which affect renal excretion and the urinary concentrations of the forbidden drugs, some drug testing programs require that the pH and specific gravity be within a specified range and may require holding the testee to provide additional urine that fulfills the criteria. For example the excretion of many nitrogen-containing drugs depends on urine pH. If the urine is alkaline, certain stimulants are excreted less, therefore a testing program may not accept a sample if the pH is 7.5 or greater. Another example is that of dilution by diuretics or excessive liquid intake, such that 1.005 may be the lowest acceptable specific gravity. These parameters may be measured with a dipstick on the urine remaining in the container after all bottles have been sealed, thus excluding the possibility of contamination by the dipstick. In some drug testing programs the specific gravity is not measured at the collection site. Some laboratories measure specific gravity and/or creatinine before proceeding with the analysis and, they may use this data to determine if the sample is valid. The laboratory must receive a sufficient volume to complete all tests. Most protocols define a minimum acceptable volume.

For the additional protection of the testee, many organizations require the division of the urine sample into two parts, A and B, to be sealed individually. The laboratory ordinarily receives both, saves the B intact, analyzes the A and reports the results to the organization.

IV. Chain of Custody

To ensure that the urine tested suffered no contamination, tampering, or mislabeling, the chain of custody begins at the collection site and ends with the final report. The sample is handled at first only by the testee until sealed, then by collection site officials, transportation personnel and laboratory technicians. The control system must guarantee integrity of the specimens from the moment of submission of the urine until the conclusion of the analysis. Each transfer must be documented, including within-laboratory transfers. Experienced couriers are recommended for the transportation of samples to the laboratory. A chain of custody form and manifest are initiated at the collection site. The laboratory checks the custody form, examines the package for evidence of tampering and then accepts custody of the package. After opening the package the samples are inspected individually and checked

against the manifest. At this juncture the laboratory initiates its internal chain of custody. Once the chain is initiated, only qualified laboratory personnel may work with the sample and no unauthorized visitors may be in the work area. On request the laboratory must be able to give exact documentation on details such as where a certain sample was located at a given time and the identity of the person handling the sample at the time in question. The samples should be stored at +4°C or colder in a locked area.

V. Analytical Approach

Because of the consequences to lives, careers and reputations which follow reports of positive analytical findings, urinalysis for banned drugs must be done using methods that give indisputable evidence. A screening test divides the samples into two categories: a large group of analytically negative samples, and a smaller group that requires further analysis (confirmation test). In the latter case, the screening data indicate that a banned substance or its metabolites may be present and provide tentative identification. The principles of decision theory have been used to calculate the predictive value of screening and confirmation tests [14]. Confirmatory tests are time-consuming, complex and have one principal goal: they provide data for the final, unequivocal identification of the banned substance or metabolites. In addition, confirmatory tests are used to confirm the identity of the biological sample (e.g. that the urine used for the second analysis is the same as the one used for the first analysis), to exclude clerical errors and to confirm that the analysis is reproducible.

A. Sample preparation

Glassware must be scrupulously clean. False positive results have been traced to soap residues [4]. To avoid contamination with phthalates nothing but glass or teflon should come in contact with the sample. The purity of the solvents and reagents should be appropriate to the analysis. Sample preparation is specifically designed in an attempt to optimize the detection of each chemical class of substance. If the drugs and metabolites are excreted as conjugates (sulfates or glucuronides), a hydrolysis step is necessary prior to extraction. Compared to acid hydrolysis, enzymatic hydrolysis is usually less destructive to deconjugated products. Polar compounds containing hydroxyl, keto, acid, or amine functions are usually converted to less polar and more volatile derivatives. The most common derivatizations are trimethylsilylation, trifluoroacylation and methylation. Many unconjugated nitrogen-containing compounds may be extracted with diethyl ether at pH > 12, separated by GC and detected with a nitrogen phosphorus selective detector (NPD). Acidic compounds, e.g. barbiturates and benzodiazepines, require extraction at appropriate acidic pH. Amphoteric compounds, such as morphine, are extracted most efficiently at their isoelectric point with polar solvents such as ether and propan-2-ol. Conjugated nitrogen compounds (beta-blockers, opiates, hydroxylated phenylalkylamines) may be extracted after hydrolysis with diethyl ether, derivatized by trimethylsilylation and/or trifluoroacylation, separated by GC, and detected by NPD. Many diuretics are acidic compounds and are extracted at pH < 2. Because

of their low volatility and thermolability they are not amenable to GC analysis without derivatization. Diuretics may be screened for by HPLC with UV detection or by GC and/or by GC-MS.

B. Screening tests

Most of the screening tests performed currently are based either in immunoassay or chromatographic techniques.

Immunoassay (IA). This is the most commonly employed screening test [3]. A wide variety of IA-based methods have been developed and automated. One common feature of IA is the utilization of antibodies with specificity for the drug and/or metabolite and closely related substances. Since the antibodies cross-react with substances which are similar in structure to the target substance, the analytical results are neither unambiguous nor strictly quantitative and must be confirmed. A variety of interferences have been described (e.g. Ref. 15).

Chromatography. The different kinds of chromatography (e.g. TLC, GC, LC) are basically separation techniques used to resolve complex biological mixtures. With appropriate physico-chemical detection and with strict standardization of experimental parameters identification and quantitation can be achieved.

Thin-layer chromatography (TLC) is a common screening test for drugs of abuse. After an extraction at controlled pH, a tentative identification of drugs can be made based on physico-chemical characteristics (R_f values) and color reactions.

High performance liquid chromatography (HPLC) is ideally suited for thermolabile and polar substances. It is most commonly used in a modification known as reverse phase chromatography. The conditions may be adjusted to solve a wide range of analytical separation problems. The available choice of detection — UV/visible, fluorescence, electro-chemical, chemical detection and MS — will allow sensitive group or even substance detection.

Gas chromatography will separate thermostable drugs that are sufficiently volatile to be eluted from the analytical column. Fused silica capillary columns provide the necessary high resolution to separate extremely complex mixtures or biological matrices. Polar substances need derivatization prior to GC. With element-specific detectors (e.g. NPD) or in combination with a MS, selective and sensitive detection of a wide variety of drugs is possible.

C. Confirmation tests

In confirmation tests the unknown and the standard undergo simultaneous sample preparation from extraction to derivatization followed by analysis. Evidence of the presence of a compound includes multiple measures of similarity, such as instrumental analysis data and relative amounts (or concentrations) of multiple urinary metabolites.

Although several approaches may be used to confirm the presence of substances in urine, gas chromatography-mass spectrometry (GC-MS) is currently the method of choice for unambiguous identification. It is the combination of two techniques, where the MS is used as a detector for the GC. GC separates the components of a

mixture and introduces them one by one into the mass spectrometer, which records a mass spectrum or 'fingerprint' of each isolated component. Properly performed GC-MS analysis unequivocally identifies the compound, not just the drug class or chemical family. For the confirmatory analysis an extract from a new aliquot of urine is prepared using the same protocol as for the screening procedure, or using an appropriate alternative.

GC provides two elements of identification: RT and RRT. The GC retention time (RT) of a compound is the time elapsed between injection of the extract into the inlet and appearance of the compound at the outlet. It is reproducible under equal operating conditions. If an unknown has the same RT as the standard it may be the same substance or it may be a different one. If an internal standard is present in both the unknown extract and the standard extract, one can calculate the relative retention time (RRT) as: $RT(\text{compound})/RT(\text{internal standard})$. The RRT is more reproducible than the RT. If an unknown has a RRT different from the RRT of the standard it is a different substance. The main determinant of RT and RRT is column polarity. Another means of characterizing a substance is retention index as originally described by Kovats [16] and later modified and improved by various authors [17]. This is useful because it allows comparison with the scientific literature.

Mass spectrometry is a very powerful technique but many of its capabilities cannot be realistically applied on a large scale for routine work. The two most frequently employed ionization techniques are electron impact and chemical ionization; The electron impact mode is commonly used to obtain a full scan. Chemical ionization is particularly useful to confirm the molecular weight of a substance.

Most mass spectrometers can give an interpretable full scan spectrum with less than 1 ng of material. However even greater sensitivity can be achieved by monitoring only a few characteristic ions of the suspected compounds in the selective ion monitoring (SIM) mode. Data collected in the SIM mode may not be considered as sufficient proof of positive results by the most demanding chemists, nevertheless SIM data are commonly presented to document a positive finding and this is acceptable to many regulatory bodies and scientists. If the analysis is based on SIM data, the certainty is greatly enhanced if more than one characteristic substance is found in the sample, e.g. the parent drug and a metabolite. In addition if SIM data are used it is important to demonstrate equivalence between ion ratios for the sample and a standard. Furthermore one should demonstrate that the ion of interest dominates its region by monitoring the ions immediately preceding and following it. This shows that the pertinent ion is not derived from the preceding ion and that the following ion is present in the proper ratio to the pertinent ion.

Unambiguous identification is accomplished by matching the RRT and spectra of the identified substances with those of authentic reference standards concurrently extracted from spiked urines (positive quality control) or certified positive cases. The reference spectra are contained in a mass spectral library. Such a library should be developed in each laboratory by analyzing derivatized compounds or their metabolites under comparable operating conditions. If reference standards of metabolites are not available, clinical studies may be performed by administration of the parent drug to man followed by timed urine collections and the resulting urines used as positive quality control samples.

VI. Quality Assurance

Quality assurance is '....planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality' [18,19]. Quality assurance encompasses quality control (QC) and quality assessment. The overall aim is to ensure that the analytical results are of sufficient accuracy for their intended application.

The goal of quality assurance in drug testing is to minimize and document the probability of false positive and false negative results, as well as to document compliance with good laboratory practices (GLP) and the requirements of regulatory agencies. Quality is a team work ethic which materializes in various forms in all aspects of the work. Quality assurance may be implemented by a designated senior scientist and manager. Some laboratories refer to such individuals as QA officers.

In the context of large drug testing programs, for example those operated by governments, sport authorities and military establishments, quality is monitored by accreditation and proficiency testing programs that establish requirements of competence and equivalency for the protection of the organization conducting the testing and of the individuals tested. Usually the requirements cover personnel needs and qualification and material resources (e.g. instruments) and analytical capabilities specific to a finite list of substances at specific concentrations. Compliance is documented by on-site inspection and written reports. Failure to comply results in immediate restriction of the work. The proficiency testing aspect of the program utilizes biological samples prepared to contain known amounts of specific banned substances. The samples are sent to the laboratory in the blind (unknown to the laboratory) or open (known to the laboratory) mode. Proficiency testing programs document the strengths and deficiencies of the participating laboratories and provide objective data for between laboratory comparisons [20,21].

Critical review of QC data and optimization of QC design should be a constant and integral part of the ongoing work in a drug testing laboratory. Each laboratory maintains written Standard Operating Procedures (SOP) which describe each protocol in unique, detailed and mandatory terms. Technicians are expected to adhere to the SOP. Laboratories should utilize internal QC biological samples either blind (to those doing the analyses) or open. Good quality control procedures monitor all possible sources of error and provide for rapid isolation of the problem. At least ten percent of the samples in a batch are related to QC. Such procedures assess: clerical accuracy, sample preparation recovery, chromatographic performance, mass spectral sensitivity, overall assay detection threshold and precision in quantitation. Examples of independent cross checks are overlapping successive batches of QC samples or requiring verification by two persons. Record-keeping should include QC data and the description of measures taken to correct actual problems.

VII. Interpretation of Test Results

A. Pharmacokinetics

In theory complete knowledge of the pharmacokinetics of a drug enables a precise prediction of the concentration of drug in urine at various times after drug ad-

ministration. In practice, however, many relevant variables cannot be known and the predictions become relatively gross estimates. Nevertheless knowledge of the principles of pharmacokinetics enables the person responsible for interpreting the results of a drug test (the interpreter) to provide the most complete and accurate report and assessment.

The concentration of drug in plasma at various times after intravenous administration drug administration is determined by the dose and clearance (a pharmacokinetic variable that encompasses half-life and volume of distribution) [22]. The concentration in urine may also be estimated if clearance of drug and water are known. If the drug is orally administered the model must include bioavailability and the rate of absorption. Sophisticated pharmacokinetic models can also account for multiple and variable doses and dosage intervals [22,23]. Drugs with long half-lives and/or large volumes of distribution may be detectable in small amounts in urine for months. Examples of studies which provide useful pharmacokinetic data are: marijuana [24–26], cocaine [23,27], amphetamine [28]. Reviews [3,6,8] provide additional references.

Many of the relevant variables are rarely known therefore the interpreter evaluates the pattern of excretion of drug and metabolites as determined by clinical studies. One must consider the sensitivity of the assays since it is a major determinant of detection times. Clearly a large dose will result in a positive test for a longer time than a small dose. Multiple closely spaced doses will result in drug accumulation, an increase in total body burden and longer detection times. For example urine collected within the first few hours after cocaine administration contains both cocaine and benzoylecgonine, while urine collected several hours later will not contain detectable amounts of cocaine [27]. The principal metabolite of tetrahydrocannabinol (11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid) is usually detectable for 1 or 2 days after single exposure, however, multiple exposures may lead to a positive test for 2 or more weeks after cessation [25]. In addition passive exposure to marijuana smoke may result in sufficient absorption to result in detectable amounts of marijuana metabolites in urine [29].

Single doses of many anabolic steroids result in a positive test for 1–3 days, while multiple doses result in positive tests for many days or weeks. Steroids that are formulated in oil and administered by injection may be detectable for several months.

B. Drug metabolism

The majority of drugs are lipophilic and undergo oxidative metabolism resulting in more polar, ionizable metabolites which are either eliminated as such or after conjugation. Alterations in the rate and extent of drug metabolism influences the elimination half-life and clearance of drugs and as a consequence, the concentrations of parent drug and of metabolites in blood and in urine.

About 90% of all oxidative metabolic reactions are catalyzed by the cytochrome *P*-450 enzyme system present in endoplasmic reticulum of liver cells, therefore this enzyme system plays a central role in the metabolism of lipophilic drugs such as stimulants, beta-blockers, steroids, opioids and other drugs.

Comprehensive knowledge of the metabolism of each banned substance is essential to the interpretation of drug testing results. For example the ingestion of some

types of poppy seeds results in morphine in the urine [30]. Since morphine is a major metabolite of heroin, the detection of morphine is compatible with administration of heroin or the ingestion of poppy seed products. One way to clarify this situation is to analyze the urine for 6-monoacetylmorphine, which is a metabolite of heroin and not a metabolite of morphine and not found in poppy seeds [31].

C. Drug interactions

The best studied drug interactions known to markedly influence drug or metabolite concentrations in body fluids are inductions and inhibitions of enzymatic reactions catalyzed by cytochrome *P*-450. Induction results in a decrease in half-life (increase in clearance) and more rapid elimination from the body. Furthermore induction often changes the pattern of metabolite elimination. Substances well known to induce the *P*-450 system include drugs such as barbiturates, glutethimide, carbamazepine, ethanol and phenytoin (hydantoins) and environmental chemicals such as polycyclic aromatic hydrocarbons (3-methylcholanthrene, benzo[*a*]pyrene) and polychlorinated aromatic hydrocarbons (polychlorinated biphenyls).

The inhibition of oxidative drug metabolic reactions by relatively unspecific inhibitors like cimetidine, chloramphenicol or sulfonamides will increase drug half-life and increase blood levels of the inhibited drug. This results in a decrease in the concentration of both parent drug and metabolites in urine and therefore the ability to detect drug use. Furthermore, the individual may experience enhanced drug effects and/or toxicity.

A number of clinically significant drug interactions resulting from concomitant administration of cimetidine with drugs such as caffeine, beta-blockers, barbiturates, morphine and ethanol have been described [32]. The mechanism of inhibition is an interaction of cimetidine with the heme iron in cytochrome *P*-450 [33]. Like cimetidine, the anabolic steroid stanozolol interacts with cytochrome *P*-450 and is a potent inhibitor of cytochrome *P*-450-catalysed reactions [34]. Depending on the particular isoenzymes that are inhibited stanozolol could alter the elimination of other anabolic steroids.

Interactions involving drugs that influence the pH and water metabolism of the body alter the excretion rates of many banned drugs. The urinary excretion of many drugs is markedly influenced by urine pH [22,28,35,36]. The excretion of basic drugs (e.g. amphetamine) decreases as the pH of urine increases. Bicarbonate has been used to rapidly change the excretion rate of basic drugs and thereby reduce the likelihood of a positive test. Similarly the excretion of acidic drugs (e.g. some diuretics) is retarded by low urinary pH. Other drugs influence the excretion of anionic substances. For example, probenecid and related substances temporarily decreases the tubular excretion of anabolic steroids, penicillins, indomethacin and others. Some drug users attempt to avoid detections by adding adulterants directly to urine [15].

The state of hydration of an individual, by its effect on the water content of urine, markedly influences the concentration of drugs. Indeed excessive intake of fluids is commonly used to dilute the urine and lower the concentration of drugs. Similarly diuretics have been used to rapidly dilute the urine. Some testing programs routinely measure the specific gravity of urine to provide some insight into this problem.

Measuring the urinary creatinine and reporting the concentration of drug per mg of creatinine is another technique of factoring for the effect of dilution.

Consideration of the pharmacokinetics, pharmacodynamics, metabolism and drug interactions leads to the conclusion that it is very difficult to answer the question of whether or not an individual was under the influence of the drug at the time the sample was taken. Given sufficient analytical data and using tenable assumptions, the interpreter may be able to offer a reasonable opinion on this question.

VIII. Documentation of Results

The analytical report should be limited to statements of fact so as not to be confused with opinion. Therefore, where necessary, two distinct documents should be provided. The analytical results and other observations are normally documented in the analytical report, whereas matters of opinion should appear in a letter which accompanies that report.

A. Content of the analytical report

The analytical report must provide sufficient information to enable the recipient to identify the individual(s) from whom the sample(s) described in the report originated. To avoid transcription errors all code numbers should be checked and double-checked by two persons. A complete description of the testing occasion is the best way to prevent such errors.

The report will contain chain-of-custody information including the date and time of arrival of the samples in the laboratory so that the analytical report may be linked to other chain-of-custody documentation. The integrity of the samples will be documented by including statements that the samples were sealed and, where appropriate, recording the seal numbers or other identifying features. The type(s) of assay performed must be stated either explicitly or, where an accepted protocol has been established, implicitly.

Qualitative results. Either the absence of the substances or the presence of the chemical entities identified in the individual samples must be clearly stated. Examples of appropriate statements might be:

'No substance banned by the [insert name of organization] was found in any of the samples'

or

'The sample coded 2345A was found to contain [insert proper name] as described in the details attached. No substance banned by the [insert name of organization] was found in any of the other samples.'

The proper name should be the IUPAC name of the chemical entity identified or if the identified substance is a parent drug (as distinct from a metabolite of that drug) then the International Non-Proprietary Name (INN) might be used instead. Under some circumstances the use of the IUPAC name is cumbersome. In these cases, it may be convenient to use the INN.

It is customary for supporting data to be included such as chromatographic data and GC-MS data obtained from the sample specified and, in addition, data on corresponding reference standards (or reference urines obtained from suitable excretion studies). The reference data would normally be obtained after the specified sample has been analyzed with suitable precautions to exclude the possibility of contamination. A sufficient description of the analytical methods used should be presented to enable the chromatographic and mass spectral data to be interpretable.

Quantitative results. Many drug testing programs define a particular concentration of drug or metabolite as the dividing value for reporting a urine result positive or negative. This value, which is often referred to as the cutoff, represents an administrative decision on the part of the program. The cutoff may be considerable greater than the detection limit (lowest concentration that can reliably be detected) of the assay. Cutoff concentrations may apply to the screening test and the confirmation test. For example, the IA cutoff for cocaine in the US Department of Defense program is 300 ng/ml and the corresponding cutoff for benzoylecgonine by GC/MS is 150 ng/ml [4]. This leads to the terminology 'analytical positive', which refers to a sample containing detectable amounts of drug, but which is reported or considered negative by the program.

If the quantitative results are greater than the cutoff, at a minimum the sample is reported positive, or more informatively, substance detected. It is more informative to report that the substance was detected at a concentration greater than the cutoff. Clearly, the concentration must exceed the cutoff by more than the experimental variation. In all other cases, the mean of the measured values should be stated together with an estimate of the precision, e.g. relative standard deviation (coefficient of variation) or confidence interval for the measurements.

If quantitative results are being reported and the substances under investigation are present in concentration which are less than the cut-off value but greater than the detection limit, then the results should indicate that fact in the format of: substance detected, not greater than X (where X is the cutoff defined by the program). In this case the sample is positive by analytical criteria, but negative by administrative criteria. It is informative to report the detection limit of the laboratory for that specific substance at that time. Do not report the substance as being not present or even not detected.

B. Supplemental information and opinion

Avoid including in the analytical report irrelevant findings since they might be misinterpreted or confusing. Similarly, do not include in the analytical report the finding of endogenous or even ubiquitous substances except and unless the analytical protocol so demands, e.g. where concentration limits are imposed and/or where administration of a substance which is indistinguishable chemically from an endogenous substance is suspected, e.g. testosterone.

Any additional information or statements of opinion may be expressed in a separate document, e.g. a covering letter. Examples of information which might be relevant include:

- (a) Highlighting a declaration which has accompanied the samples such as a

medical certificate, or drug declaration by the individual who has provided the sample where this is relevant and the laboratory data supports that statement.

(b) Where a chemical entity has been found and detailed in the analytical report and the presence of this substance is considered to be a characteristic metabolite of a present drug subject to control, then an appropriate statement should be given with supporting literature references, e.g. '11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid is a metabolite of THC' or 'the presence of 19-norandrosterone (3-alpha-hydroxy-5-alpha-estran-17-one) is considered to be evidence of the administration of the anabolic steroid nandrolone'.

No statement about the intent of the individual providing the sample should be made. The laboratory should restrict its report to the sample provided (other than as described above) It is generally unwise to make any unsolicited comments about the time when the last administrations might have occurred since there is unlikely to be any supporting information, e.g. dose taken, size of individual, metabolic characteristics of individual available to the laboratory at that time.

IX. Acknowledgements

We thank the following organizations and entities for supporting this work: Centre National de la Recherche Scientifique, Service Central D'Analyse (FR); the United States Olympic Committee, the National Collegiate Athletic Association (USA), the Norwegian Confederation of Sport (NOR) and the British Sports Council (GBR).

VIII. References

- 1 Catlin DH, Kammerer RC, Hatton CK, Sekera MH, Merdink JL. Analytical chemistry at the games of the XXIIIrd Olympiad in Los Angeles, 1984. *Clin Chem* 1987;33:319-327.
- 2 Council of Europe. Anti-doping Convention. Council of Europe, Strasbourg, France, 1989;1-19.
- 3 Hawks RL. Analytical Methodology. In: Hawks RL, Chiang NC, eds. Urine testing for drugs of abuse. NIDA Res Monog 73. DHHS Pub. No. (ADM) 87-1481, Washington, DC: Supt. of Documents, U.S. Govt. Print. Office 1986;30-43.
- 4 Irving J. Drug testing in the military — technical and legal problems. *Clin Chem* 1988;34(3):637-640.
- 5 National Institute on Drug Abuse. Mandatory Guidelines for Federal Workplace Drug Testing Programs. *Federal Register*, 1988;53(69):11970-11989.
- 6 Substance-Abuse Testing Committee. Critical issues in urinalysis of abused substances: Report of the substance-abuse testing committee. *Clin Chem* 1988;34(3):605-632.
- 7 Willette RE. Drug testing programs. In: Hawks RL, Chiang NC, eds. Urine testing for drugs of abuse. NIDA Res Monog 73. DHHS Pub. No. (ADM) 87-1481, Washington, DC: Supt. of Documents, U.S. Govt. Print. Office 1986;13-20.
- 8 Catlin DH, Hatton CK. Use and abuse of anabolic steroids and other drugs for enhancement of athletic performance. *Adv Int Med* 1990;36:381-405.
- 9 Masse R, Ayotte C, Dugal R. Studies on anabolic steroids. I. Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine. *J Chromatogr* 1989;489:23-50.
- 10 Brooks RV, Jeremiah G, Webb WA, Wheeler M. Detection of anabolic steroid administration to athletes. *J Steroid Biochem* 1979;11:913-917.
- 11 Donike M, Barwald KR, Klostermann K et al. Nachweis von exogenem Testosteron (The Detection of Exogenous Testosterone). In: Heck H, Hollmann W, Liesen H et al., eds. Sport: Leistung und Gesundheit. Köln, Kongressbd. Dtsch. Sportarztekonferenz, 1982;293-298.

- 12 Chamberlain RT. Legal issues related to drug testing in the clinical laboratory. *Clin Chem* 1988;34(3):633-636.
- 13 Dubin CL. Commission of inquiry into the use of drugs and banned practices intended to increase athletic performance. Canadian Government Publishing Services, Ottawa, Canada. ISBN 0-660-13610-4. pp. 139-144.
- 14 Spiehler VR, O'Donnell CM, Gokhale DV. Confirmation and certainty in toxicology screening. *Clin Chem* 1988;34(8):1535-1539.
- 15 Mikkelsen SL, Ash KO. Adulterants causing false negatives in illicit drug testing. *Clin Chem* 1988;34(11):2333-2336.
- 16 Kovats E. Gas-chromatographische charakterisierung organischer verbindungen. 1. Retentionsindices aliphatischer halogenide, alkohole, aldehyde und ketone. *Helv Chim Acta* 1958;41:1915-1932.
- 17 Evans MB, Haken JK. Recent developments in the gas chromatographic retention index scheme. *J Chromatogr* 1989;472:93-127.
- 18 ISO 9000. Quality management and quality assurance standards — Guidelines for selection and use. Geneva: International Standards Organization. 1987;I-III and 1-6.
- 19 Uldall A. Quality Assurance in clinical chemistry. *Scand J Clin Lab Invest*. 1987;(suppl 187)47:1-94.
- 20 Davis KH, Hawks RL, Blanke RV. Assessment of laboratory quality in urine drug testing: A proficiency testing pilot study. *J Am Med Assoc* 1988;260(12):1749-1754.
- 21 Hansen HJ, Caudill SP, Boone J. Crisis in drug testing: results of CDC blind study. *J Am Med Assoc* 1985;253:2382-2387.
- 22 Catlin DH. Pharmacokinetics. In: Bevan JA, Thompson JH, eds. *Essentials of Pharmacology*, Third Edition, Philadelphia: 1983:46-61.
- 23 Chow MJ, Ambre JJ, Tsuen IR, Atkinson AJ, Bowsher DJ, Fischman MW. Kinetics of cocaine distribution, elimination and chronotropic effects. *Clin Pharmacol Ther* 1985;38:318-324.
- 24 Bastiani RJ. Urinary cannabinoid excretion patterns. In: Agurell S, Dewey WL, Willette RE, eds. *The cannabinoids: chemistry, pharmacologic and therapeutic aspects*. New York: Academic Press, 1984:263-280.
- 25 Law B, Mason PA, Moffat AC, Gleadle RI, King LJ. Forensic aspects of metabolism and excretion of cannabinoids following oral ingestion of cannabis resin. *J Pharm Pharmacol* 1984;36:289-294.
- 26 Wall ME, Sadler BM, Brine D, Taylor H, Perez-Reyes M. Metabolism, disposition and kinetics of delta-9-tetrahydrocannabinol in man and women. *Clin Pharmacol Ther* 1983;34:352-363.
- 27 Ambre J, Tsuen IR, Nelson J, Belknap S. Urinary excretion of cocaine, benzoylecgonine and ecgonine methyl ester in humans. *J Anal Tox* 1988;12:301-306.
- 28 Beckett AH, Salmon JA, Mitchard M. Relationship between blood levels and urinary excretion of amphetamine under controlled and acidic and under fluctuating urinary pH value using amphetamine-¹⁴C. *J Pharm Pharmacol* 1969;21:251-258.
- 29 Cone EJ, Johnson RE. Contact highs and urinary cannabinoid excretion after passive exposure to marijuana smoke. *Clin Pharmacol Ther* 1986;40:247-256.
- 30 Bjervet K, Johnson J, Nilsson A, Schuberth J. Morphine intake from poppy seed food. *J Pharm Pharmacol* 1982;34:798-801.
- 31 Mule SJ, Casella GA. Rendering the "poppy seed defense" defenseless: identification of 6-monoacetylmorphine in urine by gas chromatography/mass spectrometry. *Clin Chem* 1988;34:1427-1430.
- 32 Murray M. Mechanisms of the inhibition of cytochrome P-450-mediated drug oxidations by therapeutic agents. *Drug Metab Rev* 1987;18:55-82.
- 33 Rendic S, Kajfez F, Ruf HH. Characterization of cimetidine, ranitidine and related structures interaction with cytochrome P-450. *Drug Metab Dispos* 1983;11:137-142.
- 34 Rendic S, Ruf HH. Interaction of stanozolol with cytochrome P-450. *Biochem Pharmacol* 1988;37:766.
- 35 Delbecke FT, Debackere M. The influence of diuretics on the excretion and metabolism of doping agents. II. Phentermine. *Arzneim-Forsch Drug Res* 1986;36:134-137.
- 36 Delbecke FT, Debackere M. The influence of diuretics on the excretion and metabolism of doping agents. III. Etilamfetamine. *Arzneim-Forsch Drug Res* 1986;36:1413-1416.

WADA Technical Document – TD2003LCOC

| | | | |
|------------------|-------------------|-----------------|-----------------|
| Document Number: | TD2003LCOC | Version Number: | 1.2 |
| Written by: | WADA Project Team | Approved by: | |
| Date: | June 5, 2003 | Effective Date: | January 1, 2004 |

LABORATORY INTERNAL CHAIN OF CUSTODY

There are two parts involved in the chain of custody for an individual *Sample*. Both components must be maintained in the Laboratory as part of its testing records. The external record is initiated at the collection site and ensures that the *Samples* and the results generated by the Laboratory can be unequivocally linked to the athlete. The Laboratory Internal Chain of Custody records are maintained within the Laboratory to record the testing process and the location of the *Sample* during testing.

The Laboratory Internal Chain of Custody is documentation (worksheets, logbooks, forms, etc.) that records the movement of *Samples* and *Sample Aliquots* during analysis. A Laboratory Internal Chain of Custody does not require a separate form. Within the Laboratory, the Laboratory Internal Chain of Custody shall be a continuous record of individuals in possession of the samples or *Sample Aliquots*. When not in an individual's possession, it should be documented that the *Sample* or *Aliquot* is within a controlled zone (Ref *International Standard* for Laboratories 5.4.3.2). The *Sample* or *Aliquot* must be in an individual's possession when in an uncontrolled or unsecured area of the laboratory. The entry into the Laboratory Internal Chain of Custody should be completed at the time that any change of possession occurs. The Laboratory Internal Chain of Custody must contain the name or initials of the individual, date of transfer, and the purpose of the transfer of possession. The individual's complete signature/name should appear in the documentation at least once.

A chain of custody is required for both "A" and "B" *Sample* bottles and every *Aliquot* prepared for a testing procedure. In the case of *Samples*, the Laboratory Internal Chain of Custody should record all movement from receipt in the Laboratory through storage and sampling to disposal. In the case of *Aliquots*, the Laboratory Internal Chain of Custody should record all movement from preparation through analysis. When a group of *Samples* is aliquotted for testing, a batch *Aliquot* Laboratory Internal Chain of Custody document for screening and/or confirmation may be used in lieu of an individual *Aliquot* Laboratory Internal Chain of Custody.

Any forensic corrections that need to be made to the document should be done with a single line through and the change should be initialed and dated by the individual making the change. No white out or erasure that obliterates the original entry is acceptable.

The chain of custody, along with relevant testimony from individuals documented on the chain of custody documents, should provide a complete record of the *Sample* or *Aliquot* location.

fragment ions $C_3H_6O^+$ and $C_3H_8N^+$ both have the rounded mass 58 but the exact calculated mass of 58.04186 for $C_3H_6O^+$ and 58.06567 for $C_3H_8N^+$. Neither mass fragments can be separated by conventional (low resolution) mass spectrometry but only by using high resolution MS with a resolution of 2500. Thus in practical terms, in this example the instrument (HRMS) can be set to detect only the signal of the mass of 58.04186 for $C_3H_6O^+$, and all masses differing by more than 0.0024 masses, such as 58.06567 for $C_3H_8N^+$, will be discriminated. Based on this fundamental physical principle HRMS analysis of AAS steroids and their metabolites isolated from urine reduces the biological background and increases the signal-to-noise ratio, yielding a much higher selectivity in screening and confirmation.

24.5.4 Detection of endogenous anabolic androgenic steroids

24.5.4.1 Indirect detection methods

The misuse of testosterone by athletes is also tested by GC-MS analysis of urinary extracts. However, the method reveals only the presence of testosterone and its ratio to epitestosterone. The mass spectrometrical data alone indicate whether testosterone originates exogenously (doping) or whether it was produced endogenously. In 1983 Donike *et al.* developed a method to calculate urinary excreted testosterone by a ratio to 17-epitestosterone. Both isomeric steroid hormones are excreted mainly as glucuronides which are enzymatically hydrolyzed before GC-MS analysis. The urinary testosterone/epitestosterone ratio (T/E ratio) represents a relatively constant factor within an individual and alterations under physical exercise have not been noted. Exogenous application of testosterone results in an increase in the urinary concentration of testosterone glucuronide, whereas epitestosterone glucuronide is not influenced. Based on measurements of large reference groups Donike proposed a T/E ratio of 6:1 as a marker to handle a urine specimen suspicious for testosterone misuse. An increased T/E value ($T/E > 6$) is not immediately considered as a positive sample. Following the WADA rule the athlete has to be further investigated and it has to be determined that the increased value is not caused by physical or pathological conditions. In practice this requires several test samples of the athlete and evaluation of previous tests in order to establish the athlete's individual T/E reference values (subject-based reference values). The test sample is considered positive when the tested T/E ratio clearly exceeds the subject-based reference values ($> \text{mean} + 3 \text{ standard deviations}$) of the athlete. In addition to the T/E ratio the testosterone and epitestosterone concentrations as well as the concentrations of the main testosterone metabolites are assessed.

Doping with dihydrotestosterone (DHT) became public knowledge after the Asian Games in 1994 when seven athletes were tested positive for DHT misuse. The criteria for DHT doping are also based on statistical methods and population-based

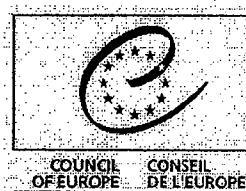
fragment ions $C_3H_6O^+$ and $C_3H_8N^+$ both have the rounded mass 58 but the exact calculated mass of 58.04186 for $C_3H_6O^+$ and 58.06567 for $C_3H_8N^+$. Neither mass fragments can be separated by conventional (low resolution) mass spectrometry but only by using high resolution MS with a resolution of 2500. Thus in practical terms, in this example the instrument (HRMS) can be set to detect only the signal of the mass of 58.04186 for $C_3H_6O^+$, and all masses differing by more than 0.0024 masses, such as 58.06567 for $C_3H_8N^+$, will be discriminated. Based on this fundamental physical principle HRMS analysis of AAS steroids and their metabolites isolated from urine reduces the biological background and increases the signal-to-noise ratio, yielding a much higher selectivity in screening and confirmation.

24.5.4 Detection of endogenous anabolic androgenic steroids

24.5.4.1 Indirect detection methods

The misuse of testosterone by athletes is also tested by GC-MS analysis of urinary extracts. However, the method reveals only the presence of testosterone and its ratio to epitestosterone. The mass spectrometrical data alone indicate whether testosterone originates exogenously (doping) or whether it was produced endogenously. In 1983 Donike *et al.* developed a method to calculate urinary excreted testosterone by a ratio to 17-epitestosterone. Both isomeric steroid hormones are excreted mainly as glucuronides which are enzymatically hydrolyzed before GC-MS analysis. The urinary testosterone/epitestosterone ratio (T/E ratio) represents a relatively constant factor within an individual and alterations under physical exercise have not been noted. Exogenous application of testosterone results in an increase in the urinary concentration of testosterone glucuronide, whereas epitestosterone glucuronide is not influenced. Based on measurements of large reference groups Donike proposed a T/E ratio of 6:1 as a marker to handle a urine specimen suspicious for testosterone misuse. An increased T/E value ($T/E > 6$) is not immediately considered as a positive sample. Following the WADA rule the athlete has to be further investigated and it has to be determined that the increased value is not caused by physical or pathological conditions. In practice this requires several test samples of the athlete and evaluation of previous tests in order to establish the athlete's individual T/E reference values (subject-based reference values). The test sample is considered positive when the tested T/E ratio clearly exceeds the subject-based reference values ($> \text{mean} + 3 \text{ standard deviations}$) of the athlete. In addition to the T/E ratio the testosterone and epitestosterone concentrations as well as the concentrations of the main testosterone metabolites are assessed.

Doping with dihydrotestosterone (DHT) became public knowledge after the Asian Games in 1994 when seven athletes were tested positive for DHT misuse. The criteria for DHT doping are also based on statistical methods and population-based



Strasbourg, 31 July 2006

T-DO (2006) 29

Anti-Doping Convention (T-DO)

Meeting of the Advisory Group on Science (T-DO SCI) Strasbourg, 11 July 2006

Meeting report

1. Opening of the meeting

Michael PETROU (Cyprus), Chair of the Advisory Group, welcomed the participants (see List of participants in Appendix 1).

The representatives of WADA, as well as the Holy See, Luxembourg, the Netherlands and Switzerland, apologised for their absence.

2. Adoption of the agenda

The agenda was adopted as set out in Appendix 2.

3. Examination of the Draft List of Prohibited Substances for 2007 and recommendations to WADA

The Chair recalled that the present draft of the Prohibited List for 2007 had been circulated as document T-DO (2006) 22. This document includes the draft list as well as the list of modifications.

S1. Anabolic agents

Additional investigation in the event of a T/E ratio higher than four

Many delegates expressed their concern regarding the high cost of isotopic ratio analysis and stated that these analyses, and the additional investigation in cases of T/E ratio higher than four, constituted a significant additional workload, not only for the accredited laboratories, but also for the Anti-Doping Organisations.

Frans DELBEKE (Belgium – Flemish Community) explained that the World Association of Anti-Doping Scientists (WAADS) conducted a survey on the results of T/E analysis. 25 out of 33 accredited laboratories replied, representing 130,018 samples. 3265 of these samples produced an adverse analytical finding in 2005. Among these, 955 (29%) of the AAF had $4 < T/E < 6$. These samples had therefore to be confirmed either by IRMS or by reviewing the results of any previous test(s) or

conducting subsequent test(s). Only 3 of the 955 samples have been confirmed (2 by IRMS and 1 by follow-up study), but not all laboratories have IRMS and also the outcomes of follow-up or previous tests are not always known by the laboratories. However, only 2 of the 789 samples analysed by IRMS contained testosterone or its precursors.

With regard to cases with $T/E > 10$ which had been analysed with IRMS, the same survey provided the following results:

10 < T/E < 15: 11 confirmed, 14 not confirmed

15 < T/E < 20: 7 confirmed, 2 not confirmed

$T/E > 20$: 26 confirmed, 1 not confirmed

Many experts expressed the desire to be provided with more data and information on the rationale behind this threshold of 4:1. Moreover, given that reservations have been expressed on the validity of the IRMS method, scientific background for its use would also be appreciated.

The Group:

- *recommended that WADA maintains the threshold of (T/E) requesting additional investigations at 4 in 2007;*
- *expressed its concerns regarding the relevance of this value with regard to the necessary workload and the limited efficiency;*
- *urged WADA to gather and publish data:*
 - a) *on positive results identified for the different classes of T/E values, from both the laboratories and the NADOs;*
 - b) *on the current research on the detection method for exogenous testosterone;*
 - c) *on the cost of the decision to lower the threshold requesting additional investigation from 6 to 4;*
 - d) *on other information (intelligence of suspected misuse, deterrence effect of these analyses) which may be useful for deciding to keep this threshold on the list;*
- *declared its intention to review its position for the List 2008, in light of the data available;*
- *recommended that WADA continues to support research on analytical methods to detect exogenous testosterone;*
- *requested the secretariat of the Monitoring Group to distribute surveys or questionnaires among the States parties in order to provide the expected information before the discussion on the List 2008, in the event that WADA cannot commit itself to releasing it;*
- *drew the WADA secretariat's attention to the formatting of the title "Other Anabolic Agents, including, but not limited to:", which should be numbered with a "2."*

S3 Beta – 2 Agonists

The Group discussed the addition of procaterol to the list of exceptions that require an Abbreviated Therapeutic Use Exemption when administered only by inhalation, and found it logical.

The Group:

- *agreed with the addition of procaterol as proposed by WADA.*

S5 Diuretics and other masking agents

Cecilia RODRIGUEZ (Spain) stated that the problems related to the lack of references to the thresholds in the List, will increase with the footnote which makes reference to the thresholds and sub-threshold without mentioning them explicitly. The problem of threshold which are not enacted in official documents (but, for example, are in circular letters of recommendation as is the case of GCS) may open the door to appeals in countries where the List has to be published as a legal provision.

She also reported possible problems which may occur in the future with the entry into force of the Unesco Convention and the adoption of the List by Unesco and the Monitoring Group of the Council of Europe.

Stanislas FROSSARD (Secretariat of the CoE) took note of the issues, and recommended that these issues be discussed within the Advisory Group on Legal Issues, whose next meeting will take place on 31 August 2006. He also stated that the note mentioning the substances which are subjected to analytical thresholds (in other standards) will be added to the List submitted to the Monitoring Group.

The Group thoroughly discussed the alpha-reductase inhibitors (e.g. finasteride and dutasteride). Taking note of a study on finasteride by Geyer et al¹ (from the Institute of Biochemistry, German Sport University, Cologne, Germany), the Group decided after this discussion to keep the alpha-reductase inhibitors on the List. Considering the risk of error and accidental use without TUE, the Group decided to recommend their inclusion on the list of "Specified Substances".

The Group discussed the comment "*A Therapeutic Use Exemption (for diuretics) is not valid if an Athlete's urine contains a diuretic in association with threshold or sub-threshold levels of a Prohibited Substance(s)*" and considered the case of the detection of a diuretic in a sample with a level of 19-norandrosterone below the level of 2 ng/ml, which is natural. The Group however concluded that in such cases, the identification of exogenous origin of such substances could be detected and that a TUE for diuretic would be valid. The Group therefore decided not to recommend amendment of the note.

The Group:

- *recommended that WADA includes finasteride and dutasteride on the list of Specified Substances;*
- *requested the Advisory Group on Legal Issues to prepare a draft decision to clarify the relation between the list adopted by the Monitoring Group and the list adopted under the Unesco Convention, in order to prevent conflict.*

M1 Enhancement of oxygen transfer

The group discussed the wording of this category of methods.

The Group:

- *suggested replacing "use" by "administration" in point a.:*
 - a. *Blood doping, including the administration of autologous, homologous or heterologous blood or red blood cell products of any origin.*

¹ Geyer, H., Gorijs, I., Mareck, U., Thevis, M., Schanzer, W. (2006) Investigation about the effects and the detection of finasteride.

M2 Chemical and physical manipulation

The Group discussed the interpretation of the meaning of “legitimate acute medical treatment”.

Klaus MÜLLER (Germany) explained that as long as the infusion is decided on the basis of diagnosis, following the consideration of a patient’s situation, it will be considered as a “legitimate acute medical treatment”. However, in cases where a whole team systematically receives infusion after competition, it would not be seen as “legitimate acute medical treatment”.

Michael Petrou mentioned the case of discipline of athletes in sports with weight categories, where it sometimes occurs that athletes receive intravenous infusions in order to compensate for dehydration and/or nutritional deficiency caused by extreme weight loss practices, and raised the question whether such practices should not be prohibited more explicitly.

S6 Stimulants

Frans Delbeke commented on pseudoephedrine, and stated that since this substance has been removed from the List he has observed many cases of pseudoephedrine misuse. He mentioned the case of an athlete who admitted the ingestion of 320 mg in one day. Misuse of this stimulant appears to be quite common in cycling. A high level of cathin is often detected in the samples of these athletes, which suggests that the presence of cathin may be a metabolite of pseudoephedrine. He stated that he would suggest the reintroduction of pseudoephedrine on the List, with an analytical detection threshold at 25 µg/ml.

Luis HORTA (Portugal) and Günter GMEINER (Austria) also reported that they had taken note of numerous cases of misuse of pseudoephedrine, in certain sports. Luis Horta also referred to a recently published article by Hodges et al², which underlined the potential performance enhancing properties of pseudoephedrine.

Katia COLLOMP (France) suggested that WADA should promote comparative studies on the physiological effects of the different types of ephedrine, as well as their dosage.

Klaus Müller underlined that this issue highlights the more general problem of the implications of removing a substance from an open list. He underlined the sensitivity of the decision to remove a substance and the importance of providing very accurate results or studies before taking such a decision.

Hans COOMAN (Belgium), also proposed to put stimulants on the List of Prohibited Substances in and out-of-competition. The rationale for this is that athletes, in certain sport, use stimulants for training or recreational purposes.

The Group:

- ***recommended that WADA puts pseudoephedrine back on the Prohibited List, as a “Specified Substance” and urged WADA, if this is done, to draw the attention of the sport community on this change in the List.***
- ***recommended that WADA includes the stimulants in the List of Prohibited Substances at All Times (in and out-of-competition).***
- ***recommended that WADA removes the second part of the last paragraph on stimulants, in order to make the following sentence: “A stimulant not expressly mentioned as an example under this section should be considered as a specified substance”.***

² Hodges, K., Hancock, S., Currell, K., Hamilton, B., and Jeukendrup, A.E. (2006) Pseudoephedrine enhances performance in 1500-m runners, *Medicine & Science in Sports & Exercise*, 38 (2), pp 329-333.

S9 Cannabinoids

Classification of cannabinoids in "Substances Prohibited in Particular Sports"

Michael STOW (United Kingdom) expressed the position of his States which is in favour of removing cannabinoids from the List.

Hans Cooman expressed the position of Belgium which would be in favour of including cannabinoids in substances prohibited in particular sport.

Michael Petrou stated that cannabinoids are placed on the List based on their capacity to indirectly enhance performance for some athletes by reducing pre-competition anxiety and promoting euphoric mood state. And also that he considers the inclusion of Cannabinoids in the List of Substances Prohibited in Competition as justified.

The Group also mentioned the number of AAF related to cannabinoids, the slow extraction of the substance which makes it difficult to distinguish recent and less recent consumption, the political issues underlying the presence of cannabinoids on the list and agreed that the right place to challenge this old and much disputed issue would be the World Anti-Doping Conference in 2007 if the criteria for including Substances and Methods on the Prohibited List are changed.

The Group:

- *took note of the proposal of the delegates from Belgium to move the cannabinoids to the category P3 (substances prohibited in particular sports) and the position of the UK to remove them from the List.*

S9 Glucocorticosteroids

The delegates from Belgium criticised the current solution which had little sense, given that athletes who wish to misuse Glucocorticosteroids can allege dermatological problems or use a TUE. The threshold of 30 ng/ml also appeared to be much too high.

Representatives of France and Portugal recognised that the present situation is unsatisfactory.

Cecilia Rodriguez complained about the lack of description of the substances covered by this class, as well as the lack of provision defining the detection threshold (except a recommendation letter to the laboratories).

The Group:

- *asked WADA to keep the Glucocorticosteroids on the List;*
- *recommended that WADA considers:*
 - *including an open list of substances as examples;*
 - *lowering the reporting level;*
- *expressed its concerns regarding the present misuse of Glucocorticosteroids.*

P1 Alcohol

The Group:

- *recommended that WADA states that the threshold values mentioned are related to blood analysis.*

Specified Substances

The Group:

- *recommended that WADA completes the line on Beta-2 agonists with "except clenbuterol and salbutamol above 1000 ng/ml".*
- *recommended the inclusion of Pseudoephedrine*
- *recommended the inclusion of finasteride and dutasteride*

4. Artificially-induced hypoxic conditions

Michael Petrou explained that this item was placed on the revised version of the Agenda after it was received from WADA consultation from its Ethical Issues Review Panel. In order to launch the discussion with background information on previously adopted decisions, Stanislas Frossard presented the previous declaration adopted by the Monitoring Group on this issue.

*Declaration on the use of altitude rooms/hyperbaric chambers
adopted by the Monitoring Group at its 11th Meeting on 30 – 31 March 2000 in Strasbourg:*

As forbidding these techniques is not the best solution, the Group suggests warning those concerned of the risks of these processes:

High-altitude training is permitted for athletes, and it cannot be banned for specific reasons: some nations/athletes are normally located at high altitudes. Artificial environments mimicking the main consequence of high altitude (lowering of the partial pressure of oxygen, i.e. the percentage of oxygen in the inspired air) could theoretically be banned, because they are unnatural, and can be controlled.

Contrary to natural high altitude, artificial means (hypoxic chambers) are not limited to conditions corresponding to several thousand meters above sea level: their pressure or oxygen content can theoretically be expanded to unnatural, dangerous or even lethal measures.

On the other hand, the real benefit in performance enhancement will certainly depend on further conditions: while enhanced performances have been observed under specific circumstances, they may lack under other ones.

Different technical concepts play an additional role: hypobaric chambers similar to high geographical altitude provide low air pressure and lower oxygen content per inhaled volume simultaneously, whereas other chambers lower only the oxygen content (lower percentage than the normal 20%). The physiological consequences or risks of those different conditions, as well as the influence on performance, may differ.

In general, these considerations must assume technical perfection. Serious risks could occur, if the predetermined, physiologically justified parameters could not be technically guaranteed.

But the possibility of obtaining similar effects by natural (although more expensive and more time-consuming) means, should be discouraged for at least unreasonably extended use, but these methods should not be prohibited for the meantime.

Stanislas Frossard also summarised the relevant discussion of the Advisory Group held in April 2005:

Many participants to the meeting expressed the need for safety standards for the commercialised hypoxic equipments, as well as the need for adequate education, so that those "training methods" do not pose a safety hazard. Some ethical concerns were also discussed (health risks, and in particular mental health risks, equity between countries, risk of abuse or misuse, medical supervision ...).

Following the presentations and extensive discussions, the Advisory Group on Science did not recommend including artificial altitude training methods as a prohibited doping method. As such methods are artificial, they may raise some ethical issues. Such methods, designed to lower oxygen content in blood, mimic natural high altitude conditions. If such methods should be used, it should only be under controlled parameters (altitude, duration, exposure) and under medical supervision.

The Group recognised that these methods have a performance enhancing potential and underlined some health risks (i.e. inappropriate use and quality/safety of equipment). The Group expressed ethical concerns related to the fact that the method is artificial.

The WADA Ethical Committee, in its recently released new contribution on the analysis of the compliance of these methods with the spirit of sport, concluded that hypoxic chambers may be seen as not complying with the spirit of sport, as defined in the introduction of the Code.

Luis Horta expressed his doubts with regard to the criteria of passivity of the athlete which has been developed by the ethical committee, as this would also challenge methods like electro stimulation, air conditioning, baths, etc.

Many experts took the floor to raise concerns about the inclusion of these methods on the List. At present, there are 3 criteria for allowing the inclusion of prohibited substances and methods on the List, but do not make this inclusion compulsory.

Warning letters from groups of scientists, although they do not represent the scientific community in general, should also be taken into consideration.

Richard L. HILDERBRAND (USA) reported that many of the physicians attending the recent American College of Sport Medicine meeting made comments against WADA controlling hypoxic chambers.

The Group, however, underlined that being against prohibition should not be interpreted as promoting this kind of method.

The Group:

- *took note that there is, at present, no support to prohibit the hypoxic chambers through their inclusion on the prohibited list;*
- *considered that, at present, all arguments are not in favour of prohibition;*
- *expressed concerns about the negative consequences of the use of the methods and invited WADA, the sport organisations and the public authorities to express warnings against the risks of hypoxic chambers and to reconsider the issue at a later date, where appropriate;*
- *asked the secretariat to invite the Bioethics Committee to give an additional independent opinion from experts on the ethical criteria (compliance of hypoxic chambers with the spirit of sport as defined in the Code).*

5. Other matters

The secretariat informed delegates that the report of the previous meeting had been released and apologised that its adoption had not been put on the agenda of the Advisory Group, as it was unclear at the time of the adoption of the agenda whether the report would be available in both languages. This report will be submitted directly to the Monitoring Group in November. However, comments from participants can be sent to the Secretariat and will be taken into consideration with the approval of the Chair of the Group.

6. Next meeting

The next meeting of the Group will take place in spring 2007. The exact date may have to be co-ordinated with the other Advisory Groups and will therefore be released at the Monitoring Group meeting.

7. Close of the meeting

The Chair thanked all participants and closed the meeting.

Appendix 1

List of participants

AUSTRIA / AUTRICHE

Dr Günter GMEINER, Head of Doping Control Lab, ARC Seibersdorf Research GMBH,
A- 2444 SEIBERSDORF
Tel. (43) 50 550 3539 Fax (43) 50 550 3566
E-mail: guenter.gmeiner@arcs.ac.at

BELGIUM / BELGIQUE

Flemmisch Community / Communauté flamande

Prof Frans DELBEKE, Head of Doping Control Laboratory, DoCoLab, Technologiepark 30,
B- 9052 ZWYNAARDJE
Tel. (32) 9 331 32 90 Fax (32) 9 331 32 99
E-mail : frans.delbeke@ugent.be

Dr. Hans COOMAN, Medical Doctor Team Health and Sports, Ministry of Flanders, Arenbergstraat 9,
B- 1000 BRUSSEL
Tel. (32) 2 553 34 83 Fax (32) 2 553 36 50
E-mail: hans.cooman@cjsm.vlaanderen.be

CYPRUS / CHYPRE

Dr Michael PETROU, (*T-DO SCI Chair / président du T-DO SCI*), Member,
National Anti-Doping Committee, Cyprus Sport Organisation, 48 Agapinoros str., CY- 2034 NICOSIA
Tel. (357) 996 35 062 Fax (357) 223 17 173
E-mail: petrou.m@cytanet.com.cy

DENMARK / DANEMARK

Mr Torben HOFFELDT, Head of Anti-Doping Section, Ministry of Culture, Nybrogade 2,
DK- 1203 COPENHAGUE K
Tel. (45) 3392 9822 Fax (45) 3391 3388
E-mail: tho@kum.dk

FINLAND / FINLANDE

Dr. Olli J. HEINONEN, M.D., PhD, Finnish Anti-Doping Association, Paavo Nurmi Centre,
University of Turku, Kiinamyllynkatu 10, FIN- 20520 TURKU
Tel. (358) 4 058 65 157 Fax (358) 2 250 26 10
E-mail: olli.heinonen@utu.fi

FRANCE

Mme Rochanak MIRFENDERESKI, Pharmacien, Cellule Dopage, Ministère de la Jeunesse, des Sports et
de la Vie associative, 95 avenue de France, F- 75013 PARIS
Tel. (33) 1 40 45 94 15 Fax (33) 1 40 45 97 56
E-mail: rochanak.mirfendereski@jeunesse-sports.gouv.fr

Mme Katia COLLOMP, Pharmacien LNDD, 143 avenue Roger Salengro,
F- 92290 CHATENAY MALABRY
Tel. (33) 1 46 60 28 69 Fax (1) 46 60 30 17
E-mail: direction@lndd.com

GERMANY / ALLEMAGNE

Prof Dr R. Klaus MÜLLER, Director, Institute fo Doping Analysis, Dresdner str. 12,
D- 01791 KREISCHA
Tel. (49) 35206 206 11 Fax (49) 34292 757 40
E-mail: rkmueeller.leipzig@t-online.de

PORTUGAL

Prof Dr Luis HORTA, IDP- Laboratorio de Análises e Dopagem, Av. Prof Egas Moniz,
(Estadio Universitario), P- 1600 LISBOA
Tel. (351) 21 79 60 245 Fax (351) 21 79 77 529
E-mail: luis.horta@idesporto.pt

ROMANIA / ROUMANIE

Prof Dr Graziala Elena VAJIALA, President, National Anti-Doping Agency, 37-39 Basarabia blvd,
Sector 2, BUCHAREST
Tel/Fax (40) 21 311 55 41
E-mail: g.vajiala@anad.gov.ro

Mrs Mia LAMOR, Director, Doping Control Laboratory, National Anti-Doping Agency, Basarabia blvd
37-39, Sector 2, BUCHAREST
Tel/Fax (40) 21 311 47 03
E-mail: m.lamor@anad.gov.ro

SLOVENIA / SLOVENIE

Dr Josko OSREDKAR, President, National Anti-Doping Commission, Olympic Committee of Slovenia,
Celovška 25, 1000 LJUBLJANA
Tel. (386) 1 23 06 008 Fax (386) 1 23 06 026
E-mail: josko.osredkar@kcclj.si

SPAIN / ESPAGNE

Dr Cecilia RODRIGUEZ BUENO, Subdirectora General de Deporte y Salud,
Consejo Superior de Deportes, c/ Greco s/n, E- 28770 MADRID
Tel. (34) 91 589 68 75/76 Fax (34) 91 544 81 22
E-mail: cecilia.rodriguez@csd.mec.es

Dr Agustin Francisco RODRIGUEZ CANO, Director, Laboratorio de Control de dopaje,
Consejo Superior de Deportes, c/ Greco s/n, E- 28770 MADRID
Tel. (34) 91 589 68 90 Fax (34) 91 543 72 90
E-mail: agustinf.rodriguez@csd.mec.es

TURKEY / TURQUIE

Dr Rüstü GÜNER, Member of the Anti-Doping Commission, Turkish Football Association,
Ankara University, School of Medicine, Department of Sports Medicine, Dikimevi, ANKARA
Tel. (90) 532 272 01 50 Fax (90) 312 362 97 82
E-mail: guner@diyalup.ankara.edu.tr

UNITED KINGDOM / ROYAUME-UNI

Mr Michael STOW, Anti-Doping Information Officer, UK Sport, 40 Bernard Street,
UK- LONDON WC1N 1ST

Tel. (44) 207 211 51 73

Fax (44) 207 211 52 48

E-mail: michael.stow@uksport.gov.uk

UNITED STATES / ETATS-UNIS

Dr Richard L. HILDERBRAND, Science Director, US Antidoping Agency, 1330 Quail Lake Loop,
Suite 260, COLORADO SPRINGS, CO

Tel. (1) 719 785 2030

Fax (1) 719 785 2029

E-mail: rhilderbrand@usantidoping.org

APOLOGIZED / EXCUSES

Holy See / Saint-Siège

Luxembourg

Switzerland / Suisse

WADA

COUNCIL OF EUROPE

Sport Department, Directorate of Youth and Sport

European Youth Centre, 30 rue Pierre de Coubertin, F- 67000 STRASBOURG

M. Stanislas FROSSARD, Administrator

Tel. (33) 3 90 21 53 76

Ms Diane PENEAU, Secretary

Tel. (33) 3 88 41 35 80

Fax: (33) 3 88 41 24 29

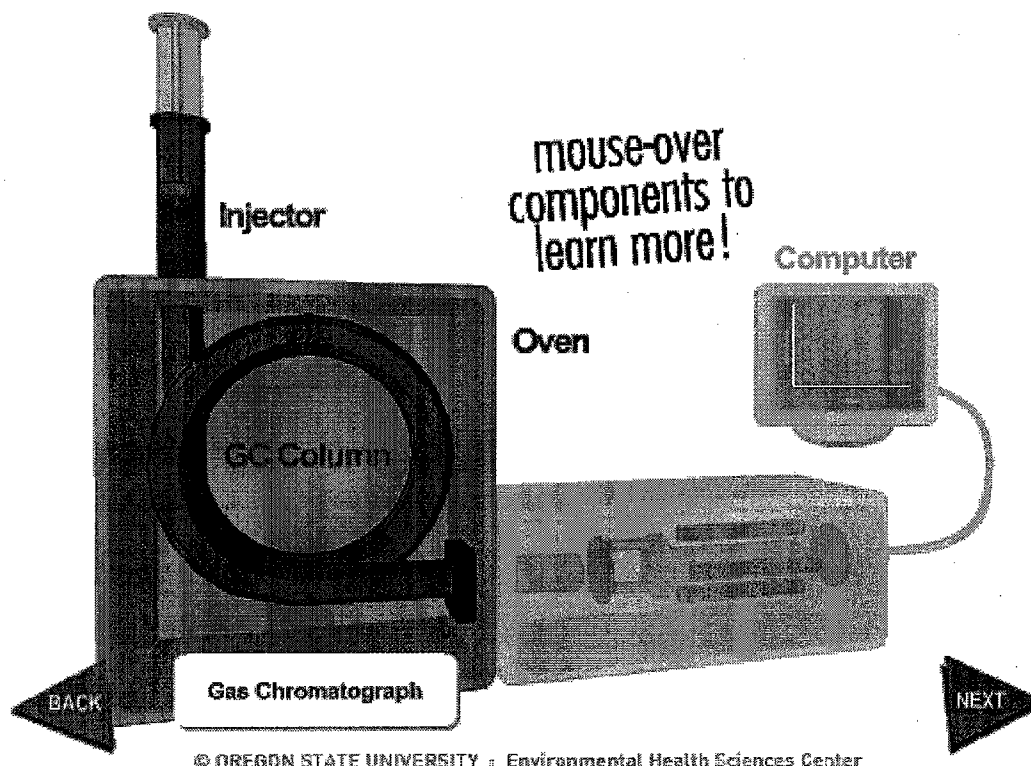
E-mail: diane.peneau@coe.int Internet: <http://www.coe.int/sport>

Appendix 2

Agenda

1. Opening of the meeting
2. Adoption of the agenda T-DO (2006) 23 rev
4. Examination of the Draft List of prohibited substances and recommendations to WADA
T-DO (2006) 22
4. Artificially-induced hypoxic conditions T-DO (2006) Inf 8
5. Other matters
6. Next meeting
7. Close of the meeting

GCMS: How Does It Work?



How does the GCMS work?

The GCMS instrument is made up of two parts. The gas chromatography (GC) portion separates the chemical mixture into pulses of pure chemicals and the mass spectrometer (MS) identifies and quantifies the chemicals.

The GC separates chemicals based on their volatility, or ease with which they evaporate into a gas. It is similar to a running race where a group of people begin at the starting line, but as the race proceeds, the runners separate based on their speed. The chemicals in the mixture separate based on their volatility. In general, small molecules travel more quickly than larger molecules.

The MS is used to identify chemicals based on their structure. Let's say after completing a puzzle, you accidentally drop it on the floor. Some parts of the puzzle remain attached together and some individual pieces break off completely. By looking at these various pieces, you are still able to get an idea of what the original puzzle looked like. This is very similar to the way that the mass spectrometer works.

1. Gas chromatography (GC)

- Injection port** – One microliter (1 μL , or 0.000001 L) of solvent containing the mixture of molecules is injected into the GC and the sample is carried by inert (non-reactive) gas through the instrument, usually helium. The inject port is heated to 300° C to cause the chemicals to become gases.
- Oven** – The outer part of the GC is a very specialized **oven**. The column is heated to move the molecules through the column. Typical oven temperatures range from 40° C to 320° C.
- Column** – Inside the oven is the **column** which is a 30 meter thin tube with a special polymer coating on the inside. Chemical mixtures are separated based on their volatility and are carried through the column by helium. Chemicals with high volatility travel through the column more quickly than chemicals with low volatility.

2. Mass Spectrometer (MS)

- A. **Ion Source:** After passing through the GC, the chemical pulses continue to the MS. The molecules are blasted with electrons, which cause them to break into pieces and turn into positively charged particles called **ions**. This is important because the particles must be charged to pass through the filter.
- B. **Filter**– As the ions continue through the MS, they travel through an electromagnetic field that filters the ions based on mass. The scientist using the instrument chooses what range of masses should be allowed through the filter. The filter continuously scans through the range of masses as the stream of ions come from the ion source.
- C. **Detector** – A detector counts the number of ions with a specific mass. This information is sent to a computer and a **mass spectrum** is created. The mass spectrum is a graph of the number of ions with different masses that traveled through the filter.

3. Computer

- A. The data from the mass spectrometer is sent to a computer and plotted on a graph called a **mass spectrum**.

Anabolic Steroids

CYNTHIA M. KUHN

*Department of Pharmacology and Cancer Biology, Duke University Medical Center,
Durham, North Carolina 27710*

ABSTRACT

The term “anabolic steroids” refers to testosterone derivatives that are used either clinically or by athletes for their anabolic properties. However, scientists have questioned the anabolic effects of testosterone and its derivatives in normal men for decades. Most scientists concluded that anabolic steroids do not increase muscle size or strength in people with normal gonadal function and have discounted positive results as unduly influenced by positive expectations of athletes, inferior experimental design, or poor data analysis. There has been a tremendous disconnect between the conviction of athletes that these drugs are effective and the conviction of scientists that they aren't. In part, this disconnect results from the completely different dose regimens used by scientists to document the correction of deficiency states and by athletes striving to optimize athletic performance. Recently, careful scientific study of suprapharmacologic doses in clinical settings – including aging, human immunodeficiency virus, and other disease states – supports the efficacy of these regimens. However, the mechanism by which these doses act remains unclear.

“Anabolism” is defined as any state in which nitrogen is differentially retained in lean body mass, either through stimulation of protein synthesis and/or decreased breakdown of protein anywhere in the body. Testosterone, the main gonadal steroid in males, has marked anabolic effects in addition to its effects on reproduction that are easily observed in developing boys and when hypogonadal men receive testosterone as replacement therapy. However, its efficacy in normal men, as during its use in athletes or in clinical situations in which men are eugonadal, has been debated. A growing literature suggests that use of suprapharmacologic doses can, indeed, be anabolic in certain situations; however, the clear identification of these situations and the mechanism by which anabolic effects occur are unclear. Furthermore, the pharmacology of “anabolism” is in its infancy: no drugs currently available are “purely” anabolic but all possess androgenic properties as well. The present review briefly recapitulates the historic literature about the androgenic/anabolic steroids and describes literature supporting the anabolic activity of these drugs in normal people, focusing on the use of suprapharmacologic doses by athletes and clinicians to achieve anabolic effects in normal humans. We will present the emerging literature that is beginning to explore more specific mechanisms that might mediate the effects of suprapharmacologic regimens. The terms anabolic/androgenic steroids will be used throughout to reflect the combined actions of all drugs that are currently available.

I. Use of Suprapharmacologic Doses of Anabolic/Androgenic Steroids (AAS)

People have been taking testosterone to restore “vitality” since the efficacy of some hormonal component of the testes was first described by Brown-Sequard

in 1889. He reported the reversal of his own aging by self-injection of a testicular extract, thereby stimulated a flurry of experimentation into the putative anti-aging effects of testicular hormones long before the identity of testosterone was confirmed. The first use to improve athletic performance occurred shortly thereafter, in 1896. A contemporary of Brown-Sequard self-administered testicular extract, then measured his finger strength. Athletes have been using purified testosterone since it was first available (see a review of this early history in Yesalis *et al.*, 2000). The modern use of anabolic steroids in athletic competition dates from the Olympic competitions during the Cold War era. Russian athletes were putatively the first to use anabolic steroids to improve athletic performance in international competitions. Although the International Olympic Committee banned use of anabolic agents in 1964, the practice spread and probably reached its pinnacle in the athletic programs in Germany during the 1970s (Yesalis *et al.*, 2000).

Medical use of testicular extract began in the late 1800s. Clinical use of supraphysiological doses of AAS in eugonadal patients for anabolic benefit started in the 1940s. High-dose AAS regimens have been used to promote muscle deposition after burns, surgery, radiation therapy, and aging-related sarcopenia (muscle wasting). Recent uses include treating wasting in human immunodeficiency virus (HIV) and contraception (Bhasin *et al.*, 1996,1997; Amory and Bremner, 2000).

II. Anabolic Steroids

All steroids that are anabolic are derivatives of testosterone and are androgenic as well as anabolic, as they stimulate growth and function of male reproductive tract. Individual drugs vary in their balance of anabolic:androgen activity but none of the currently available drugs are purely anabolic. All the anabolic steroids currently used are derivatives of testosterone or are structural modifications of testosterone that influence its pharmacokinetics, bioavailability, or balance of androgenic to anabolic activity. These include testosterone itself, all of the derivatives that are used clinically, as well as numerous plant products that at least claim to possess anabolic actions.

The testosterone derivatives available in the United States comprise several groups: 1) endogenously produced androgens or their precursors, including testosterone and androstenedione; 2) synthetic derivatives of testosterone with altered metabolic or receptor-binding characteristics; and 3) various uncharacterized plant or animal materials. Testosterone actions represent the combination of several activities. First, it binds to the androgen receptor to exert its androgenic activity. Second, it is 5 α reduced in some target tissues (including the male urogenital tract, skin, liver, and sebaceous glands) to dihydrotestosterone (DHT), which also acts on the androgen receptor. Finally, it can be aromatized to

estradiol and exert estrogenic activities. The latter two actions are highly undesirable in anabolic drugs, 5α reduction because it decreases the ratio of anabolic:androgenic activity and aromatization because of the feminizing side effects.

Structural and pharmacokinetic properties have been reviewed extensively (Wilson, 1988,1996) and are abstracted briefly here (see Figures 1 and 2).

1. Testosterone as an injectable form, a transdermal patch, skin cream, and a micronized oral preparation

2. $17\text{-}\beta$ esters of testosterone: testosterone cypionate, propionate, enanthate, and undecanoate. Esterification at this site renders the steroid more fat soluble and delays absorption into the circulation. All but the undecanoate must be injected. Nandrolone $17\text{-}\beta$ esters also exist.

3. $17\text{-}\alpha$ derivatives (methyltestosterone, methandrostenolone, norethandrolone, fluoxymesterone, danazol, oxandrolone, stanozol). These derivatives resist metabolism in the liver, so are orally active. This modification is associated with significant hepatic toxicities.

4. Modifications of the A, B, or C rings (mesterolone, nortestosterone, methenolone, fluoxymesterone, methandrostenolone, nandrolone, danazol, nandrolone, stanozol). These modifications achieve a number of goals, including a) slow metabolism; b) enhanced affinity for the androgen receptor (19-nortestosterone); c) resistance to aromatization to estradiol (fluoxymesterone, 19-nortestosterone); and d) decreased binding of metabolites to androgen receptor (5α -reduced metabolites of 19-nortestosterone, 7α -19-nortestosterone).

Structure:activity modifications that limit either conversion to DHT and/or to estradiol partially target specific testosterone derivatives to specific activities. Agents such as fluoxymesterone and 19-nortestosterone (nandrolone) that resist aromatization lack the feminizing side effects of testosterone. 19-nortestosterone possesses another characteristic that increases its anabolic activity because its

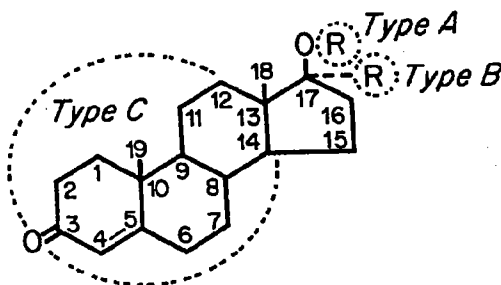


FIG. 1. Model testosterone structure. [Reprinted with permission from Wilson JD 1998 Androgen abuse by athletes. Endocr Rev 9:181-191. Copyright The Endocrine Society.]

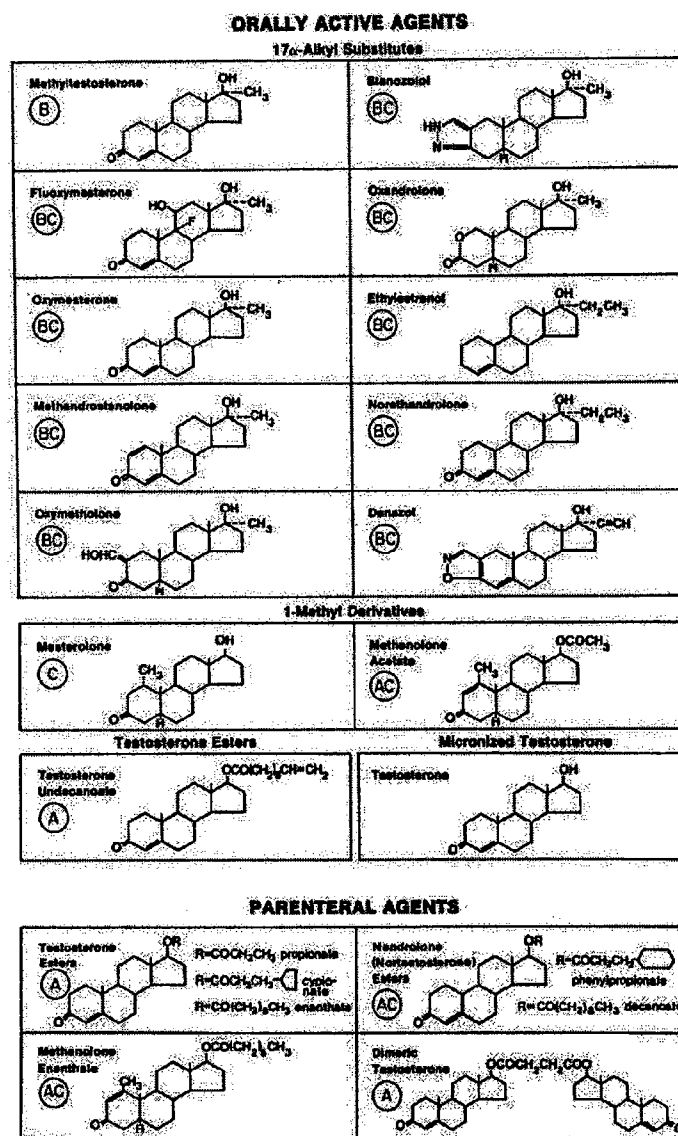


FIG. 2. Structures of several anabolic steroids. [Reprinted with permission from Wilson JD 1998 Androgen abuse by athletes. Endocr Rev 9:181-191. Copyright The Endocrine Society.]

5 α -reduced metabolite has poor affinity for the androgen receptor. Similarly, alpha-methyl-19-nortestosterone is not a substrate for 5 α reductase (Sundaram *et al.*, 1995).

Finally, a number of "natural products" that are purported to exhibit anabolic qualities are marketed freely in the United States due to the exemption of "natural products" from U.S. Food and Drug Administration regulation. Most of these are steroid precursors. Androstenedione and norandrostenedione are two widely marketed precursors. Marketers claim that they are converted into testosterone and nortestosterone (nandrolone). While a small percentage is, indeed, converted, the total amount produced is likely far below that which would have any anabolic activity in a eugonadal male. Finally, there are undefined mixtures with catchy names like "Horny Goat Weed" and "Testicular Extract" that are derived from both plant and animal materials and contain absolutely unknown ingredients.

All of the drugs listed above possess both anabolic and androgenic activities; none are absolutely selective. However, this ratio varies across a broad range. Table I shows the approximate anabolic:androgenic ratio of a number of clinically used AAS. The range is fairly narrow by clinical standards. All anabolic steroids are virilizing if administered for long enough at high enough doses.

These values are based on data collected in the 1950s and 1960s from bioassays of varying degrees of specificity and accuracy (see excellent historical review in Kochakian, 1976). Typically, the ability of a test drug to stimulate growth of a skeletal muscle and a reproductive target (prostate gland) was assessed. Two classic methods for establishing anabolic efficacy were the stimulation of growth of the levator ani muscle in the castrated rodent and stimulation of whole-body nitrogen retention in a castrated animal. Neither of these are ideal measures. The levator ani muscle may actually reflect androgenic efficacy of AAS because it can be viewed as part of the reproductive system. Its use as a bioassay for "anabolic" activity has been questioned. While the

TABLE I
Anabolic:Androgenic Ratio for Selected Anabolic Drugs

| Anabolic/androgenic steroid | Anabolic:androgenic ratio |
|----------------------------------|---------------------------|
| Testosterone, methyltestosterone | 1 |
| Methandrostenolone | 2-5 |
| Oxymetholone | 9 |
| Oxandrolone | 10 |
| Nandrolone | 10 |
| Stanozol | 30 |

nitrogen-retention assay is better, it provides an extremely indirect measure of muscle deposition.

There has been virtually no investigation of the relative anabolic and androgenic properties of AAS since the mid-1970s and none using more modern tools to assess androgen receptor activity. One major goal of this chapter is to summarize recent developments in the molecular pharmacology of androgen receptors that are opening this area up for pharmaceutical development.

III. Androstenedione

In the summer of 1998, baseball player Mark McGwire revealed that he took regular androstenedione supplements during the season that he set a new home run record. The first scientific study of the anabolic efficacy of androstenedione appeared shortly thereafter (King *et al.*, 1999). This study showed that giving modest doses to untrained men who were started on an exercise program increased testosterone only transiently at the higher (i.e., 300-mg) dose but did not improve strength. However, it did increase plasma estradiol levels, a finding that was confirmed in a later study (Leder *et al.*, 2000). That is, this study recapitulated the large number of negative studies in the literature that documented that such combinations of training and AAS were no more effective than training alone. This study has been replicated in older men using a similar design (Broeder *et al.*, 2000). In this case, users took doses recommended by supplement manufacturers while they were engaged in resistance training. The results of both studies were similar: neither younger nor older subjects who received androstenedione showed greater increases in strength than those who received placebo, although circulating lipid profiles changed in the direction of greater cardiovascular risk (low-density to high-density lipoprotein/apolipoprotein A/apolipoprotein B) ratio. Since, at most, 10–15% of a dose is converted to testosterone, it is unlikely that regimens used by athletes will prove anabolic but the research has not been conducted. No published studies report effective anabolic activity of suprapharmacologic doses of androstenedione.

IV. Do Anabolic Steroids Increase Muscle Size and/or Strength in Eugonadal Men?

The anabolic effects of restoring normal physiologic levels of testosterone in hypogonadal men are uncontested. The rise in testosterone during puberty contributes to the increase in linear growth as well as muscle deposition at that time. Increased muscle deposition clearly results when hypogonadal men receive testosterone treatment (Kopera, 1985; Wilson, 1996; Bross *et al.*, 1999). AAS also can be anabolic in men who are hypogonadal as a result of disease such as HIV or after burns (Bhasin *et al.*, 1996, 1999).

The anabolic effects of testosterone derivatives in women athletes are similarly explicable, as circulating testosterone levels of women are typically about 10% of those observed in men (Wilson, 1996). Therefore, raising female testosterone levels to those comparable to males provides supraphysiologic levels. Although there are very few published studies of muscle size and strength after AAS use in women, one report found elevations up to 30-fold of normal levels in women who were self-administering AAS (Malarkey *et al.*, 1991). There are virtually no controlled studies of AAS effects on women for obvious ethical reasons but dramatic evidence of these effects derives from the recently released results of the East German sports program of the 1970s and 1980s (Franke and Berendonk, 1997). Figure 3 shows shotput performance of a female German athlete, with the bars below indicating the periods of AAS administration. Unfortunately, women receiving AAS inevitably experience the androgenization associated with these drugs.

The benefit of anabolic steroid use for eugonadal men is far more controversial.

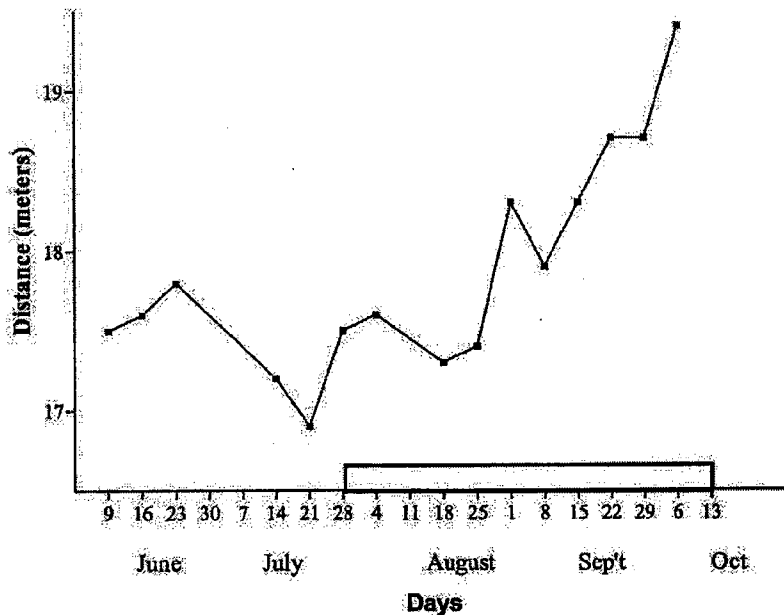


FIG. 3. Shot-put performance of woman athlete from East Germany. [Redrawn from Franke WW, Berendonk B 1997 Hormonal doping and androgenization of athletes: a secret program of the German Democratic Republic government. Clin Chem 43:1262-1279.]

For decades, scientists argued that anabolic steroids do not increase muscle mass or strength in normal men. This was based first on clinical experience that suggested that the nitrogen-retaining effects of testosterone treatment to normal men were modest and transient (Wilson, 1996). The controversy between those who state that anabolic steroids do not increase muscle mass or strength and those who believe in their effectiveness derives in part from which body of research is cited. Many critics properly cite many negative studies in which addition of testosterone or another AAS to a training regimen failed to improve performance (see reviews in Wilson, 1988; Elashoff *et al.*, 1991; O'Connor and Cicero, 1993; Friedl, 2000). They also critique the poor study design in studies conducted in athletes, including lack of placebo control, nonblinded study conditions, reliance on case studies or small study populations, lack of standardization of dose and training regimen, and the impact that expectation of benefit had on results.

These differences in study design might well play an important part in the different findings. First, nonfit people who are started on a training regimen generally experience such substantial benefit from the training regimen alone that it is difficult to show an additive benefit of AAS. The use of physiologic doses of AAS contributed to this problem. Little benefit of increasing testosterone within the physiologic range has been demonstrated in such studies. Furthermore, the dependent measure chosen to assess muscle strength is critical. Testosterone increases upper body mass differentially, so performance in tasks like weightlifting should improve more than lower-body tasks or tasks in which aerobic capacity rather than strength are assessed. As expected, the task in which increases have been reported most reliably are in the bench press (Friedl, 2000). Finally, the degree of improvement expected in such studies is generally small. Changes in performance of 1–5% are rarely statistically or clinically significant but they represent the margin of victory for elite athletes. Therefore, scientists, clinicians, and athletes all might interpret data from the same study quite differently.

Controversies about anabolic effects of AAS in animals have been similar but less intense. The same factors have entered into the outcome: gender of animals, conducting the study in trained vs. sedentary animals, dose regimen, and duration of exposure. AAS do effectively increase muscle size, protein content, and contractility in both male and female rats (Exner *et al.*, 1973; Menschikowski *et al.*, 1988; Lewis *et al.*, 1999), although negative findings have been reported (Bates *et al.*, 1987). Efficacy of AAS in trained animals has been established (Lubek *et al.*, 1984; Elashoff *et al.*, 1991; Lewis *et al.*, 1999), although different muscle beds respond differentially and slow twitch muscles improve more than fast twitch (Sachs and Leipheimer, 1988; Lewis *et al.*, 1999; Joumaa and Leoty, 2001).

V. Challenging the Conventional Wisdom: AAS Can Increase Muscle Size and Strength in Normal Men

Studies providing suprapharmacologic doses, using maximally trained athletes and testing performance in tasks like weightlifting, are mainly likely to show an effect of AAS. A recent study showing clear, statistically significant increases in muscle mass and strength after AAS administration in a proper placebo-controlled, blinded study may help put these controversies to rest. This complemented previous studies from the same laboratory demonstrating benefit in hypogonadal, HIV-infected men using the same strategy (Bhasin *et al.*, 1996,1999,2001; Strawford *et al.*, 1999).

Biochemical and anatomical studies show that AAS do significantly influence muscle morphology and biochemistry in humans. Body weight reliably increases after AAS use and part of the increase is in lean body mass, although part also reflects retention of water (see recent review in Friedl, 2000). Muscle biopsies in weightlifters reported that both the number of muscle fibers and average fiber size in the trapezius muscle were greater in AAS users than nonusers (Doumit *et al.*, 1996; Kadi *et al.*, 1999a). Controlled studies show that both the number of muscle fibers and the size of individual fibers increase with AAS treatment in animal models (Joubert and Tobin, 1989). Both of these processes depend upon activation of satellite cells within the muscle. Satellite cells contain androgen receptors (Doumit *et al.*, 1996). AAS action within these cells to stimulate proliferation may represent an important mechanism of AAS action. The specific genes that are regulated by androgens in the muscle are unknown. Muscle biopsies in AAS-using powerlifters, in comparison to drug-free powerlifters, showed increased expression of embryonic forms of myosin and the Leu-19 antigen that is expressed in developing myotubes and newly formed myonuclei. This finding supports the hypothesis that AAS trigger both hypertrophy and hyperplasia but does not elucidate the specific genes that are activated (Kadi *et al.*, 1999a,b,2000).

Increases in strength can also result not from hypertrophy or hyperplasia but from increased expression of specific elements of the contractile apparatus. Again, this has been little studied. However, a recent study (Joumaa and Leoty, 2001) began to address this phenomenon by evaluating potassium and caffeine-induced contractures. Both the magnitude of potassium-induced contractures and the rate of recovery were greater in slow-twitch muscles of animals that received training and nandrolone. The authors speculated that these results suggested changes in both the activation mechanism and recovery mechanisms that sequester calcium in the sarcoplasmic reticulum. Enhanced caffeine contractures could reflect enhanced calcium release from the sarcoplasmic reticulum or changes in the calcium sensitivity of the contractile proteins.

VI. Mechanism of Anabolic Effect in Eugonadal Men

A. ROLE OF SUPRAPHARMACOLOGIC DOSES

The mechanism by which AAS increase muscle size and strength is surprisingly confusing. Androgen receptors clearly mediate the increase in muscle size and protein synthesis in hypogonadal men and during puberty. In these situations, androgen increases net nitrogen balance, increases lean body mass, and increases the rate of muscle protein synthesis (see review by Wilson, 1996). However, it often is asserted that comparable effects are not observed in men with normal gonadal function because androgen receptors are saturated at physiologic levels of testosterone. If androgen effects are mediated by androgen receptors, which are saturated at physiologic levels of testosterone, then no additional benefit should result from providing more androgen.

Steroid regimens favored by athletes differ markedly from those used clinically to provide replacement for hypogonadal men. Athletes use suprapharmacologic doses and typically "stack" multiple drugs at total androgen doses that range from 10–100-fold above normal levels (Wilson, 1988). Typically, they take androgens in cycles of weeks, with drug holidays interspersed of weeks or months. Many athletes use "stacking" regimens that involve taking multiple agents simultaneously, and/or a pyramiding dose regimen in which doses are started low, increased, then tapered back down.

A small but expanding literature suggests that suprapharmacologic doses are effective in eugonadal men. The active hormone is probably testosterone, since 5α reductase is not present in muscle (Wilson and Gloyna, 1970). Two older studies (Griggs *et al.*, 1989; Forbes *et al.*, 1992) have been supplemented by several recent findings demonstrating increased lean body mass, muscle protein synthesis, and/or positive nitrogen balance in normal men after high doses of AAS (Bhasin *et al.*, 1996; Ferrando *et al.*, 1998; Sheffield-Moore *et al.*, 1999; Strawford *et al.*, 1999; see also review in Sheffield-Moore, 2000). The most important recent finding is the dose-response study showing that androgenic effects of testosterone saturate at fairly low doses, in contrast to measurable anabolic effects, which require considerably higher doses (Bhasin *et al.*, 1999).

B. ANDROGEN RECEPTOR IN AAS: EFFECTS ON EUGONADAL MEN

The finding that muscle hypertrophy associated with exercise is blocked by androgen antagonists (Inoue *et al.*, 1994) supports a primary role for androgen receptors in exercise-induced muscle hypertrophy. One androgen receptor has been cloned (see review by Lamb *et al.*, 2001) and while its expression varies quantitatively in muscle and reproductive tissues (Sar *et al.*, 1990; Kimura *et al.*, 1993), it is likely that this receptor mediates AAS effects in the muscle. The one study comparing the binding of a range of AAS to skeletal muscle and prostate

reported, as expected, that little tissue specificity in binding affinity was observed across a broad range in binding affinities (Saartok *et al.*, 1984). Androgen receptors are present in skeletal muscle of every mammalian species (Sar *et al.*, 1990; Takeda *et al.*, 1990). Levels of expression differ from muscle bed to muscle bed in a manner consistent with reported AAS effects on muscle strength in different tasks. For example, human muscle beds differ from each other, with expression higher in the muscles of the neck and chest girdle, in comparison to the limbs (Kadi *et al.*, 2000).

Recent studies in multiple species show that androgen receptor can be upregulated by exposure to AAS (Bricout *et al.*, 1994; Doumit *et al.*, 1996; Sheffield-Moore *et al.*, 1999; Kadi *et al.*, 2000). The induction reported in humans (Sheffield-Moore *et al.*, 1999) suggests that suprapharmacologic concentrations might be effective because they increase the population of androgen receptors upon which they can act. These findings suggest at least one potential mechanism by which high doses could elicit different effects than physiologic doses.

In summary, two aspects of androgen receptor expression can influence the magnitude of anabolic effects: variations from muscle bed to muscle bed in androgen receptor expression and induction of androgen receptor expression after treatment with AAS. These are shown schematically in Figure 4.

Recent insights into the organization of the steroid hormone receptor:DNA complex suggest an alternative explanation for the varying anabolic:androgenic ratio of AAS. Steroid hormone receptors form a "tripartite" complex between ligand, receptor, and effector that can have varying actions (Katzenellenbogen *et al.*, 1996). When steroid hormones or their analogues bind to their receptor, they form a complex that binds to DNA. However, the receptor:DNA complex also binds a group of adaptor proteins that influence the transcriptional consequences of receptor binding to DNA. These proteins function as coactivators or co-repressors to enhance or prevent activation of transcription by the receptor (Torchia *et al.*, 1998). Each drug that binds steroid hormone receptor induces a particular "shape" in the drug:receptor complex that permits a unique pattern of adapter protein association. The adapter proteins that associate influence the consequence of drug:receptor binding on transcription (Darimont *et al.*, 1998). Selectivity can derive from the drug, the receptor, or the pattern of adaptor protein expression. This model has been exploited successfully in the development of tissue-selective estrogenic compounds. Different estrogenic compounds have specific actions, depending upon the coactivator and/or co-repressor environment (see McDonnell *et al.*, this volume).

A recent commentary (Negro-Vilar, 1999) suggests that a similar approach could be considered for androgens. Up to six different coactivators that are relatively specific for the androgen receptor have been described in the literature (Yeh and Chang, 1996; Fujimoto *et al.*, 1999; Kang *et al.*, 1999; Muller *et al.*,

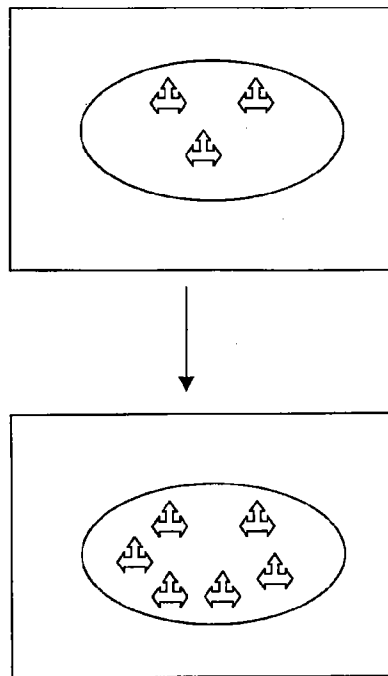


FIG. 4. Model cell containing nucleus with few (top) or more (bottom) androgen receptors. Top represents head and neck muscle relative to leg or normal individual compared to AAS-treated individual.

2000). Although tissue distribution is incompletely described, all are expressed in testis and prostate with widely varying levels of expression in other androgen target tissues. One, FHL2, is expressed highly in heart, slightly in prostate, but not elsewhere (Muller *et al.*, 2000). This coactivator is the first described that is expressed more highly in a nonreproductive tissue than in reproductive tissues. Its existence suggests that tissue-specific distribution of coactivators could theoretically contribute to the ability of different AAS agonists to vary in their ratio of actions in different tissues due to the different tissue distribution of coactivators or co-repressors. Information from a different source supports the possibility that different agonists do induce different conformations of the drug:receptor complex. An NH₂-terminal and carboxyl-terminal interaction of the androgen receptor occurs in the presence of agonist binding (Langley *et al.*, 1995). In a co-transfection system, this interaction parallels agonist activity to a degree but weak agonists like medroxyprogesterone possess agonist activity in

the absence of this interaction (Kemppainen *et al.*, 1999). However, the study of androgen receptor interactions of this type is in its infancy. It suggests that nonselective steroids like testosterone might occupy the androgen receptor in a way that produces a receptor conformation that permits binding of both general and tissue-selective co-activators (Figure 5). Model drugs with selective actions would result in a ligand:receptor conformation that permitted association only of one set of tissue-selective ligands (Figure 6).

C. ANTICATABOLIC EFFECTS OF ANDROGENS

There is also evidence to support a role for anticatabolic mechanisms in the anabolic effects of suprapharmacologic AAS regimens. A recent case report of two patients with a point mutation in the androgen receptor that rendered it inactive showed that a suprapharmacologic steroid regimen was anabolic in both individuals (Tincello *et al.*, 1997). Evidence from animal models also supports this possibility. These begin with binding studies that show that androgens can bind, albeit at low affinity, to glucocorticoid receptors (Danheive and Rousseau, 1986,1988). Such low-affinity binding would not be effective, unless extremely

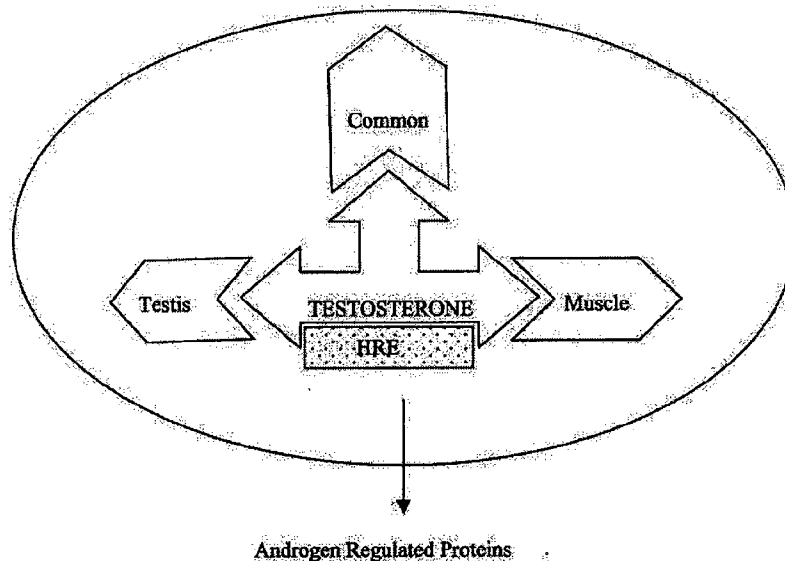


FIG. 5. Model of testosterone activation of androgen receptor (showing just one of the two ligand:receptor dimers that bind to DNA and activate transcription). HRE = hormone response element.

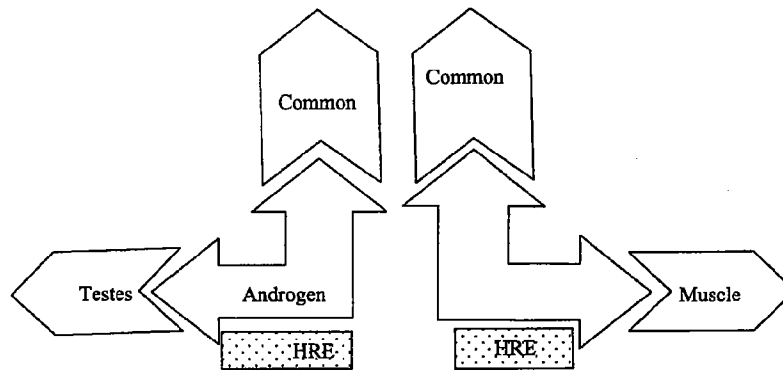


FIG. 6. Hypothetical ligand-occupied androgen receptor conformations that would allow an agonist to recruit coactivators in a tissue-specific way.

high doses of AAS were present. More directly, testosterone can block glucocorticoid-mediated induction of tyrosine amino transferase in liver just like the glucocorticoid antagonist RU486 (Danhaive and Rousseau 1986,1988). However, androgen blockade of glucocorticoid-induced muscle wasting is not observed consistently (see review by Hickson *et al.*, 1990). Furthermore, in healthy young men as well as in burn patients, the anabolic steroid oxandrolone has been shown to increase net protein synthesis without slowing protein degradation (Sheffield-Moore *et al.*, 1999; Hart *et al.*, 2001). Therefore, the specific contribution of glucocorticoid antagonism in AAS-induced anabolic effects has not been demonstrated unequivocally.

D. COMPLEMENTARY EFFECTS ON GROWTH HORMONE SECRETION AND INSULIN-LIKE GROWTH FACTOR-1 PRODUCTION

The growth hormone (GH)-insulin-like growth factor-1 (IGF-1) axis is thought to contribute to the anabolic effects of testosterone, both through androgen-induced stimulation of GH secretion and direct stimulation of hepatic production of IGF-1 (Rosenfeld *et al.*, 1994; Veldhuis and Iranmanesh, 1996). IGF-1 can stimulate skeletal muscle formation (Florini *et al.*, 1991). Increases in IGF mRNA have been reported in rats following nandrolone administration and increases in both IGF mRNA and circulating IGF-1 occur in men after testosterone treatment (Urban *et al.*, 1995; Gayan-Ramirez *et al.*, 2000) and decreases in mRNA occur when gonadal function is suppressed (Mauras *et al.*, 1998). However, none of these studies measured IGF-1 directly or established the relationship between IGF-1 and anabolic effects of the drugs.

E. COMPLEMENTARY EFFECTS OF TRAINING AND AAS

One of the challenges involved in understanding the effects of AAS in normal athletes is that many of the endpoints (e.g., muscle size, strength) are enhanced by training. Most studies of AAS action involve administration of AAS to sedentary animals, which presents a clear picture of what isolated AAS administration can achieve. Furthermore, comparing the benefits of beginning an exercise regimen and/or AAS in an unfit person provides a model for potential treatment of patient populations such as patients with HIV or the elderly. It does not replicate the environment in which AAS are most often used, which is in a highly trained athlete who is adding AAS to a rigorous exercise regimen. Clearly, exercise increases muscle mass on its own. The prospective, placebo-controlled testosterone trial in eugonadal men by Bhasin and colleagues (1996) that compared placebo, testosterone, exercise, or exercise plus testosterone showed clearly that effects of testosterone and resistance exercise were additive. Another recent study suggests a possible mechanism by which AAS use and exercise might complement each other. Resistance exercise itself increases androgen receptor mRNA and/or binding in both rodent and human muscle (Deschenes *et al.*, 1994; Bamman *et al.*, 2001). If androgen receptor number is induced in muscle by exercise, then more binding sites become available.

F. ROLE OF THE CENTRAL NERVOUS SYSTEM IN AAS EFFECTS ON STRENGTH

An increased sense of energy and wellbeing is one of the earliest and most frequently documented effects in hypogonadal men. It has been suggested that effects within the central nervous system (CNS) contribute to AAS effects on strength because AAS users feel more energetic and therefore train harder. Case reports, cross-sectional studies, and prospective, longitudinal studies show that AAS use by athletes can be accompanied by increased feelings of energy, aggressiveness, and elevated mood (Bahrke and Yesalis, 1996; Rubinow and Schmidt, 1996; Pope *et al.*, 2000). Effects of AAS typically focus on negative reports of psychotic symptoms and criminal aggressive behavior (Pope *et al.*, 1988, 2000; Uzych, 1992; Porcerelli and Sandler, 1998). However, two studies of high-dose androgen administration to normal volunteers reported increases in euphoria, energy, and sexual arousal, as well as several negative mood characteristics, including irritability, mood swings, violent feelings, and hostility (Hannan *et al.*, 1991; Su *et al.*, 1993). However, administration of supraphysiologic levels of testosterone did not change aggression as assessed with the Multi-Dimensional Anger Inventory in normal, eugonadal men (Tricker *et al.*, 1996). These few laboratory studies do not provide definitive answers to this question because they utilize controlled dosing of testosterone (although in the supraphysiologic range), employ a variety of methods for measuring aggression/

hostility, and use as subjects eugonadal men in laboratory settings rather than athletes in highly competitive settings who are self-administering even-higher doses of steroids. However, the frequency of case reports of extreme behaviors and positive findings in controlled studies suggest that AAS might influence strength through effects on behavior.

The mechanism by which the psychological effects of androgens occur is unknown. A study of cerebrospinal fluid (CSF) monoamine levels in a controlled study of high-dose methyltestosterone administration reported that levels of the serotonin metabolite 5HIAA were higher and levels of the norepinephrine metabolite MHPG were lower after methyltestosterone treatment. 5HIAA levels correlated negatively in subjects who experienced more negative mood symptoms (e.g., irritability, hostility) and higher 5HIAA in subjects who experienced increased mood symptoms such as euphoria (Daly *et al.*, 2001). The latter findings are consistent with a broad literature supporting an association between low serotonin and aggression/irritability/hostility (Lucki, 1998; Oquendo and Mann, 2000). Another study showed increases in aggression that correlated with changes in CSF dopamine metabolites (Hannan *et al.*, 1991).

Testosterone influences brain function by three mechanisms. It contributes to the differentiation of brain areas that regulate regulation of reproductive hormone secretion, sexual behavior, as well nonreproductive behaviors, including aggression (reviewed by Rubinow and Schmidt, 1996). While these organizational effects establish the anatomical basis for sex-specific behavior patterns, they do not contribute to the acute effects of AAS. Androgens also influence many neural functions through both classical genomic effects and rapid membrane effects. Androgen receptors are distributed (Simerly *et al.*, 1990; Pelletier, 2000) and likely have similar distributions in humans. AAS administration – at least in animal models – can increase androgen receptor number in some brain areas, just as it does in muscle (Lynch and Story, 2000). Effects on many neurotransmitter-specific proteins, including serotonin receptors, choline acetyltransferase, the rate-limiting synthetic enzyme for acetylcholine, and monoamine oxidase have been described (see review in Rubinow and Schmidt, 1996). These likely reflect changes in transcriptional activity but effects of suprapharmacologic doses are virtually unexplored.

Rapid membrane effects also may contribute to behavioral effects of AAS. Suprapharmacologic doses of AAS influence GABA receptor function acutely, over a timeframe that likely reflects rapid membrane rather than genomic effects. In some brain areas and model systems, AAS decrease GABA receptor function, while in others it increases it (Masonis and McCarthy, 1996; Jorge-Rivera *et al.*, 2000). Rapid changes in GABA function theoretically could contribute to disinhibition of behavior and changes in arousal like those reported in AAS users. A single recent report (Schlussman *et al.*, 2000) showed nandrolone caused

increases in corticotropin (ACTH) and corticosterone secretion acutely as well as protracted effects that were the reverse 24 hours later. This finding indicates that AAS influences at least one neuronal system related to stress and arousal exhibits through what may be both rapid and genomic effects.

Unfortunately, the question remains: do the behavioral effects of AAS influence training intensity and, therefore, muscle strength? Furthermore, although speculations abound that AAS improve neuromuscular function, this hypothesis has not been tested either.

The issue of AAS "dependence" reflects another widely publicized notion based on a small amount of data. Although not directly related to anabolic effects of AAS, a brief discussion is provided because this issue features prominently in discussion of AAS effects on behavior. Several studies report incidence of steroid "dependence" as reflected by psychological symptoms, including depressed mood, fatigue, anorexia, insomnia, restlessness, muscle and joint pain, depression, and desire to take more AAS when athletes stop using (Uzych, 1992; Bahrke and Yesalis, 1996). These reports – and the public perception that AAS use represented a public health crisis – led to the labeling of AAS as "addictive" drugs that were then scheduled by the Drug Enforcement Agency.

There are no clear data supporting the "addictiveness" of AAS use. This may reflect a lack of information or the fact that these drugs are not "addictive" in a neurobiologic sense. "Addictive drugs" must 1) be self-administered by humans and animals, 2) produce positive subjective effects, and 3) produce tolerance and dependence, manifested as a withdrawal syndrome when use stops. Other addictive drugs elicit positive subjective effects by activating the "reward" system in the brain, adaptation of which is thought to produce the gradual dysregulation of drug use (Wise, 1998). In a classical sense, anabolic steroids do not activate the reward system. They are not self-administered by animals and people cannot discriminate an injection of testosterone from placebo (see review in Lukas, 1996). It is impossible to conduct double-blind, placebo-controlled studies of long-term testosterone treatment on mood because users can usually recognize the active drug from the side effects. However, few AAS users fulfill psychiatric criteria for drug dependence (Lukas, 1996).

Nevertheless, some AAS users report positive feelings when they are taking drug and changes in mood when they stop (Su *et al.*, 1993; Lukas 1996). How does one reconcile the clinical reports with the laboratory studies? Genomic effects of AAS are delayed rather than immediate, so they would not be detected in any of the standard models of drug taking. It is possible that AAS do affect reward systems in the brain but in a delayed manner, as would be expected from a gonadal steroid, and so these effects have not been detected. Occasional reports of AAS effects on aspects of dopaminergic transmission, including upregulation of D1 receptors and increases in dopamine turnover, suggest that further explo-

ration of this possibility is warranted (Thiblin *et al.*, 1999; Kindlundh *et al.*, 2001).

VI. Other Consequences of Suprapharmacologic AAS Regimens

Androgen receptors are distributed throughout the body. Androgens affect behavior, cardiovascular function, reproduction, and other endocrine functions. Since anabolic actions are not easily dissociated pharmacologically from the other actions of testosterone derivatives, anabolic steroid use by athletes and patients inevitably is accompanied by unwanted side effects that result from the many actions of androgens in the body. During a typical high-dose paradigm, additional AAS effects occur, including 1) feedback inhibition of reproductive function, including decreased production of testosterone and sperm; 2) acne, due to stimulation of sebaceous glands in the skin; and 3) male-pattern hair distribution (Wilson, 1988). In addition, multiple effects on the cardiovascular system occur, including increased blood pressure, change in the ratio of blood lipids (decrease in HDL:LDL ratio), increased blood clotting, increased production of red blood cells, and left ventricular hypertrophy and subsequent decreased left ventricular function (Sullivan *et al.*, 1998). Extended discussion of potential mechanisms for these effects is beyond the scope of this review. However, the regular occurrence of these additional effects contradicts the common argument that AAS cannot be anabolic because androgen receptors are completely saturated at physiologic levels of androgen. The impact of these other systemic effects on the anabolic effects of AAS is unknown. Although increased production of red blood cells should theoretically improve oxygen-carrying capacity of the blood, and so the ability to do sustained work, these effects have not been documented in eugonadal men.

One final note about the use of AAS for their anabolic properties: when used in women, they produce a consistent pattern of virilizing side effects that are predictable, severe, and, in some cases, irreversible. The first published study (Strauss *et al.*, 1985) reported physical changes in the majority of a small group of AAS-using female athletes, including deepening of the voice, clitoral hypertrophy, menstrual irregularities, decreased body fat, and increased facial hair. Behavioral changes included increased libido, aggressiveness, and appetite. About half reported additional changes, including acne, breast size, and body hair distribution. A more recent study (Malarkey *et al.*, 1991) reported a 39% fall in HDL lipoprotein. All of these effects were reported more recently (Gruber and Pope, 2000) in a study involving a larger group. Some of these effects (e.g., deepening of the voice, clitoral hypertrophy) represent irreversible virilization, while others (e.g., reproductive effects, acne, blood lipids) are reversible. The consistency of these findings argues strongly that clinical trials for AAS use for anabolic purposes, as in burn patients, be conducted with great caution because there is no clinically available AAS that lacks androgenizing effects in women.

VII. Conclusions

Studies in AAS-using human subjects as well as experimental model systems have refuted the decades-old assertion that suprapharmacologic dose regimens of AAS are not anabolic in normal men or are only anabolic due to the impact of their CNS effects on motivation to train. The physiopathology of suprapharmacologic doses of AAS is clearly demonstrated and predicted by the beneficial effects on the same systems when AAS are used in hypogonadal men. However, there has been surprisingly little work on the mechanism by which these suprapharmacologic doses exert their actions or on pharmacologic strategies to distinguish beneficial (anabolic) effects from pathologic side effects on brain and heart. The recent demonstration of clinical benefits of suprapharmacologic regimens (Bhasin *et al.*, 1996,1997,1999,2001) suggests that such developments could be clinically beneficial. A recent review proposed the potential value of exploring the possible tissue specificity of protein regulators of androgen receptor function, comparable to those which have been exploited so successfully in the development of selective estrogen receptor modulators (Negro-Vilar, 1999).

REFERENCES

- Amory JK, Bremner WJ 2000 Newer agents for hormonal contraception in the male. *Trends Endocrinol Metab* 11:61–66
- Bahrke MS, Yesalis CE 1996 Psychological and behavioral effects of endogenous testosterone and anabolic-androgenic steroids. *Sports Med* 22:367–390
- Bamman MM, Shipp JR, Jiang J, Gower BA, Hunter GR, Goodman A, McLafferty CL, Urban RJ 2001 Mechanical load increases muscle IGF-1 and androgen receptor mRNA concentrations in humans. *Am J Physiol* 280:E383–E390
- Bates PCL, Chew LF, Millward DJ 1987 Effects of the anabolic steroid stanozol on growth and protein metabolism in the rat. *J Endocrinol* 114:373–381
- Bhasin SW, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Ricker R, Shirazi A, Casaburi R 1996 The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med* 335:1–7
- Bhasin S, Storer TW, Berman N, Yarasheski K, Clevenger B, Casaburi R 1997 A replacement dose of testosterone increases fat-free mass and muscle size in hypogonadal men. *J Clin Endocrinol Metab* 82:407–413
- Bhasin S, Storer TW, Javanbakht M, Berman N, Yarasheski KE, Phillips J, Dike M, Sinha-Hikim I, Shen R, Hays RD, Beall G 1999 Testosterone replacement and resistance exercise in HIV-infected men with weight loss and low testosterone levels. *J Am Med Assn* 283:763–770
- Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Sher R, Storer TW 2002 Testosterone dose response relationship in healthy young men. *Am J Physiol*, in press
- Bricout V, Germain P, Serrurier B, Guezennec C 1994 Changes in testosterone muscle receptors: effects of an androgen treatment on physically-trained rat. *Cell Mol Biol* 40:291–294
- Broeder CE, Quindry J, Brittingham K, Panton L, Thomson J, Appakondur S, Breul K, Byrd R, Douglas J, Earnest C, Mitchell C, Olson M, Roy T, Yarlagadda C 2000 The Andro

- project: physiological and hormonal influences of androstenedione supplementation in men 35 to 65 years old participating in a high-intensity resistance training program. *Arch Intern Med* 160:3093–3104
- Bross R, Javanbakht M, Bhasin S 1999 Anabolic interventions for aging-associated sarcopenia. *J Clin Endocrinol Metab* 84:3420–3430
- Daly RC, Su TP, Schmidt PJ, Pickar D, Murphy DL, Rubinow DR 2001 Cerebrospinal fluid and behavioral changes after methyltestosterone administration: preliminary findings. *Arch Gen Psych* 58:172–177
- Danhaive PA, Rousseau GG 1986 Binding of glucocorticoid antagonists to androgen and glucocorticoid hormone receptors in rat skeletal muscle. *J Steroid Biochem* 24:481–487
- Danhaive PA, Rousseau GG 1988 Evidence for sex-dependent anabolic response to androgenic steroids mediated by muscle glucocorticoid receptors in the rat. *J Steroid Biochem* 29:575–581
- Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, Fletterick RJ, Yamamoto KR 1998 Structure and specificity of nuclear receptor-coactivator interactions. *Genes Dev* 12:3343–3356
- Deschenes M, Maresch C, Armstrong L, Covault J, Kraemer W, Crivello J 1994 Endurance and resistance exercise induce muscle fiber type specific responses in androgen binding capacity. *J Ster Biochem Mol Biol* 50:175–179
- Doumit ME, Cook DR, Merkel RA 1996 Testosterone up-regulates androgen receptors and decreases differentiation of porcine myogenic satellite cells *in vitro*. *Endocrinology* 137:1385–1394
- Elashoff JD, Jacknow AD, Shain SG, Braunstein GD 1991 Effects of metabolic-androgenic steroids on muscular strength. *Ann Intern Med* 115:387–393
- Exner GU, Staudte HW, Pette D 1973 Isometric training of rats: effects on fast and slow muscle and modification by an anabolic hormone (nandrolone decanoate). II. Male rats. *Pflüger's Arch* 345:15–22
- Ferrando AA, Sheffield-Moore M, Wolf SE, Herndon DN, Wolfe RR 2000 Testosterone normalization in severe burns ameliorates muscle catabolism. *FASEB J* 14(4):A797
- Florini JR, Ewton DZ, Roof SL 1991 IGF-1 stimulates terminal myogenic differentiation by induction of myogenin gene expression. *Mol Endocrinol* 5:718–724
- Forbes GB, Porta CR, Herr BE, Griggs MRC 1992 Sequence of changes in body composition induced by testosterone and reversal of changes after drug is stopped. *J Am Med Assn* 267:397–399
- Franke WW, Berendonk B 1997 Hormonal doping and androgenization of athletes: a secret program of the German Democratic Republic government. *Clin Chem* 43:1262–1279
- Friedl KE 2000 Effect of anabolic steroid use on body composition and physical performance. In: Yesalis CE, ed. *Anabolic Steroids in Sport and Exercise*. Champaign, IL: Human Kinetics; 139–174
- Fujimoto N, Yeh S, Kang HY, Inui S, Chang HC, Mizokami A, Chang C 1999 Cloning and characterization of androgen receptor co-activator, RA55, in human prostate. *J Biol Chem* 274:8316–8321
- Gayán-Ramírez G, Rollier H, Vanderhoydonc F, Verhoeven G, Gosselink R, Decramer M 2000 Nandrolone decanoate does not enhance training effects but increases IGF-1 mRNA in rat diaphragm. *J Appl Physiol* 88:26–34
- Griggs RC, Kingston W, Jozefowicz RF, Herr BE, Forbest G, Halliday D 1989 Effect of testosterone on muscle mass and muscle protein synthesis. *J Appl Physiol* 66:498–503
- Gruber AJ, Pope HG 2000 Psychiatric and medical effects of anabolic-androgenic steroid use in women. *Psychother Psychosom* 69:19–26

- Hannan CJ, Friedl KE, Zold A, Kettler TM, Plymate SR 1991 Psychological and serum homovanillic acid changes in men administered androgenic steroids. *Psychoneuroendocrinology* 16:335–343
- Hart DW, Wolf SE, Ramzy PI, Chinkes DL, Beauford RB, Ferrando AA, Wolfe RR, Herndon DN 2001 Anabolic effects of oxandrolone after severe burn. *Ann Surg* 233:556–564
- Hickson RC, Czerwinski SM, Falduto MT, Young AP 1990 Glucocorticoid antagonism by exercise and androgenic-anabolic steroids. *Med Sci Sports Med* 22:331–340
- Inoue K, Yamasaki S, Fushiki T, Okada Y, Sugimoto E 1994 Androgen receptor antagonist suppresses exercise-induced hypertrophy of skeletal muscle. *Eur J Appl Physiol* 69:88–91
- Jorge-Rivera JC, McIntyre KL, Henderson LP 2000 Anabolic steroids induce region- and subunit-specific rapid modulation of GABA-A receptor mediated currents in the rat forebrain. *J Neurophysiol* 83:3299–3309
- Joubert Y, Tobin C 1989 Satellite cell proliferation and increase in the number of myonuclei induced by testosterone in the levator ani muscle of the adult female rat. *Dev Biol* 131:550–557
- Joumaa WH, Leoty C 2001 Differential effects of nandrolone decanoate in fast and slow rat skeletal muscle. *Med Sci Sports Exerc* 33:397–403
- Kadi F, Eriksson A, Holmner S, Butler-Browne G, Thornell LE 1999a Cellular adaptation of the trapezius muscle in strength trained athletes. *Histochem Cell Biol* 111:189–195
- Kadi F, Eriksson A, Holmner S, Thornell LE 1999b Effects of anabolic steroids on the muscle cells of strength-trained athletes. *Med Sci Sports Exerc* 31:1528–1534
- Kadi F, Bonnerud P, Eriksson A, Thornell LE 2000 The expression of androgen receptors in human neck and limb muscles: effects of training and self-administration of androgenic-anabolic steroids. *Histochem Cell Biol* 113:25–29
- Kang HY, Yeh S, Fujimoto N, Chang C 1999 Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. *J Biol Chem* 274:8570–8576
- Katzenellenbogen JA, O'Malley BW, Katzenellenbogen BS 1996 Tripartite steroid hormone receptor pharmacology: interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. *Mol Endocrinol* 10:119–131
- Kemppainen JA, Langley E, Wong C, Bobseine K, Kelce WR, Wilson EM 1999 Distinguishing androgen receptor agonists and antagonists: distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. *Mol Endocrinol* 13:440–454
- Kimura N, Mizokami MA, Oonuma T, Sasano H, Nagura H 1993 Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *J Histochem Cytochem* 41:671–678
- Kindlundh AM, Lindblom J, Bergström L, Wikberg JE, Nyberg F 2001 The anabolic-androgenic steroid nandrolone decanoate affects the density of dopamine receptors in the male rat brain. *Eur J Neurosci* 13:291–296
- King DS, Sharp RL, Vukovich MD 1999 Effect of oral androstenedione on serum testosterone and adaptations to resistance training in young men: a randomized controlled trial. *J Am Med Assn* 281:2020–2028
- Kochakian CD 1976 *Anabolic-Androgenic Steroids*. New York: Springer-Verlag
- Kopera H 1985 The history of anabolic steroids and a review of clinical experience with anabolic steroids. *Acta Endocrinol (suppl)* 271:11–18
- Lamb DJ, Weigel NL, Marcelli M 2001 Androgen receptors and their biology. *Vitam Horm* 62:199–230
- Langley E, Zhou ZX, Wilson EM 1995 Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. *J Biol Chem* 270:29983–29990

- Leder BZ, Longcope C, Catlin DH, Ahrens B, Shoenfeld DA, Finkelstein JS** 2000 Oral androstenedione administration and serum testosterone concentrations in young men. *Clin Invest J Am Med Assn* 283:779–782
- Lewis MI, Fournier M, Yeh AY, Micevych PE, Sieck PM** 1999 Alterations in diaphragm contractility after nandrolone administration: an analysis of potential mechanisms. *J Appl Physiol* 86:985–992
- Lubek BM** 1984 Contractile responses of rat lateral gastrocnemius and soleus to dianabol (17 beta-hydroxy-17-methyl-1,4androstadien-3-one) and exercise. *Steroids* 44:485–495
- Lucki I** 1998 The spectrum of behaviors influenced by serotonin. *Biol Psychol* 44:151–162
- Lukas SE** 1996 CNS effects and abuse liability of anabolic-androgenic steroids. *Annu Rev Pharmacol Toxicol* 36:333–357
- Lynch CS, Story AJ** 2000 Dihydrotestosterone and estrogen regulation of rat brain androgen receptor immunoreactivity. *Physiol Behav* 69:445–453
- Malarkey WB, Strauss RH, Leizman DJ, Liggett M, Demers LM** 1991 Endocrine effects in female weight lifters who self-administer testosterone and anabolic steroids. *Am J Obstet Gynecol* 165:1385–1390
- Masonis AE, McCarthy MP** 1996 Effects of the androgenic/anabolic steroid stanozol on GABA A receptor function: GABA-stimulated $^{36}\text{Cl}(-)$ influx and $[\text{S}]$ TBPS binding. *J Pharmacol Exp Ther* 279:186–193
- Mauras N, Hayes V, Welch S, Rini A, Helgeson K, Dokler M, Veldhuis JD, Urban RJ** 1998 Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. *J Clin Endocrinol Metab* 83:1886–1892
- McDonnell DP, Connor CE, Wijayarathne A, Chang CY, Norris JD** 2002 Definition of the molecular and cellular mechanisms underlying the tissue selective agonist/antagonist activities of selective estrogen receptor modulators (SERMs). *Rec Progr Horm Res* 57:295–316
- Menschikowski M, Jung K, Junghans P, Petzke KJ, Albrecht V** 1988 The influence of a steroid hormone and physical exercise on protein metabolism in rats. *Exp Clin Endocrinol* 92:341–348
- Muller JM, Isele U, Metzger E, Rempel A, Moser M, Pschere A, Breyer T, Holubarsch C, Buettner R, Schule R** 2000 FHL2, a novel tissue-specific coactivator of the androgen receptor. *EMBO J* 19:359–369
- Negro-Vilar A** 1999 Selective androgen receptor modulators (SARMs): a novel approach to androgen therapy for the new millennium. *J Clin Endocrinol Metab* 84:3459–3462
- O'Connor LH, Cicero TJ** 1993 Anabolic steroids: misuse or abuse. In Chulkin JS, ed. *Hormonally Induced Changes in Mind and Brain*. New York: Academic Press; 129–156
- Oquendo MA, Mann JJ** 2000 The biology of impulsivity and suicidality. *Psychiat Clin N Am* 23:11–25
- Pelletier G** 2000 Localization of androgen and estrogen receptors in rat and primate tissues. *Histol Histopathol* 15:1261–1270
- Pope HG, Katz DL** 1988 Affective and psychotic symptoms associated with anabolic steroid use. *Am J Psychiat* 145:487–490
- Pope HG, Kouri EM, Hudson JI** 2000 Effects of supraphysiological doses of testosterone on mood and aggression in normal men: a randomized controlled trial. *Arch Gen Psychol* 57:133–140
- Porcerelli JF, Sandler BA** 1998 Anabolic-androgenic steroid abuse and psychopathology. *Psychiat Clin N Am* 21:829–832
- Rosenfeld RG, Rosenbloom AL, Guevara-Aguirre J** 1994 Growth hormone (GH) insensitivity due to primary GH receptor deficiency. *Endocr Rev* 15:369–390
- Rubinow DR, Schmidt PJ** 1996 Androgens, brain and behavior. *Am J Psychiat* 153:974–984

- Saartok T, Dahlberg E, Gustafsson JA 1984 Relative binding affinity of anabolic-androgenic steroids: comparison of the binding to the androgen receptors in skeletal muscle and in prostate, as well as to sex hormone-binding globulin. *Endocrinology* 114:2100–2106
- Sachs BD, Leipheimer RE 1988 Rapid effect of testosterone on striated muscle activity in rats. *Neuroendocrinology* 48:453–458
- Sar M, Lubahn DB, French FS, Wilson EM 1990 Immunohistochemical localization of the androgen receptor in rat and human tissues. *Endocrinology* 127:3180–3186
- Schlussman SD, Zhou Y, Johansson P, Kiuru A, Ho A, Nyberg F, Kreek MJ 2000 Effects of the androgenic anabolic steroid, nandrolone decanoate, on adrenocorticotropin hormone, corticosterone and proopiomelanocortin, corticotropin releasing factor (CRF) and CRF receptor1 mRNA levels in the hypothalamus, pituitary and amygdala of the rat. *Neurosci Lett* 284:190–194
- Sheffield-Moore M 2000 Androgens and the control of skeletal muscle protein synthesis. *Ann Med* 32:181–186
- Sheffield-Moore M, Urban RJ, Wolf SE, Jiang J, Catlin DH, Herndon DN, Wolfe RR, Ferrando AA 1999 Short-term oxandrolone administration stimulates net muscle protein synthesis in young men. *J Clin Endocrinol Metab* 84:2705–2711
- Simerly RB, Chang C, Muaramatsu M, Swanson LW 1990 Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an *in situ* hybridization study. *J Compar Neurol* 294:76–95
- Strawford A, Barbieri T, Van Loan M, Parks E, Catlin D, Barton N, Neese R, Christiansen M, King J, Hellerstein MK 1999 Resistance exercise and supraphysiologic androgen therapy in eugonadal men with HIV-related weight loss: a randomized controlled trial. *J Am Med Assn* 281:1282–1290
- Strauss RH, Liggett MT, Lanese RR 1985 Anabolic steroid use and perceived effects in ten weight-trained women athletes. *J Am Med Assn* 253:2871–2873
- Su TP, Pagliaro M, Schmidt PJ, Pickar D, Wolkowitz O, Rubinow DR 1993 Neuropsychiatric effects of anabolic steroids in male normal volunteers. *J Am Med Assn* 269:2760–2764
- Sullivan ML, Martinez CM, Gennis P, Gallagher EJ 1998 The cardiac toxicity of anabolic steroids. *Progr Cardiovasc Dis* 41:1–15
- Sundaram K, Kumar N, Monder C, Bardin CW 1995 Different patterns of metabolism determine the relative anabolic activity of 19-norandrogens. *J Steroid Biochem Mol Biol* 53:253–257
- Takeda H, Chodak G, Mutchnik S, Nakamoto T, Chang C 1990 Immunohistochemical localization of androgen receptors with mono- and polyclonal antibodies to androgen receptor. *J Endocrinol* 126:17–25
- Thiblin I, Finn A, Ross SB, Stenfors C 1999 Increased dopaminergic and 5-hydroxytryptaminergic activities in male rat brain following long-term treatment with anabolic androgenic steroids. *Br J Pharmacol* 126:1301–1306
- Tincello DG, Saunders PTK, Hodgins MB, Simpson NB, Edwards CRW, Hargreave TB, Wu FCW 1997 Correlation of clinical, endocrine and molecular abnormalities with *in vivo* responses to high-dose testosterone in patients with partial androgen insensitivity syndrome. *Clin Endocrinol* 46:497–506
- Torchia J, Glass C, Rosenfeld MG 1998 Co-activators and co-repressors in the integration of transcriptional responses. *Curr Opin Cell Biol* 10:373–383
- Tricker R, Casaburi R, Storer TW, Clevenger B, Berman N, Shirazi A, Bhasin S 1996 The effects of supraphysiological doses of testosterone on angry behavior in healthy eugonadal men – a clinical research center study. *J Clin Endocrinol Metab* 81:3754–3758
- Urban RJ, Bodenbun C, Gilkison C, Foxworth J, Coggan AR, Wolfe RR, Ferrando A 1995 Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. *Am J Physiol* 269:E820–E826

- Uzych L** 1992 Anabolic-androgenic steroids and psychiatric-related effects: a review. *Can J Psychiat* 37:23–28
- Veldhuis JD, Iranmanesh A** 1996 Physiological regulation of the human growth hormone (GH)-insulin-like-growth factor type I (IGF-I) axis: predominant impact of age, obesity, gonadal function and sleep. *Sleep* 19(10 suppl):S221–S224
- Wilson JD** 1988 Androgen abuse by athletes. *Endocr Rev* 9:181–191
- Wilson JD** 1996 Androgens. In Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Goodman Gilman A, eds. *Goodman Gilman's Experimental Basis of Therapeutics*. New York: McGraw-Hill; 1441–1457
- Wilson JD, Gloyne E** 1970 The intranuclear metabolism of testosterone in the accessory organs of male reproduction. *Rec Progr Horm Res* 26:309–336
- Wise RA** 1998 Drug activation of brain reward pathways. *Drug Alcohol Depend* 51:13–22
- Yeh S, Chang C** 1996 Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. *Proc Natl Acad Sci USA* 93:5517–5521
- Yesalis CE, Courson SP, Wright JE** 2000 History of anabolic steroid use in sport and exercise. In: Yesalis CE, ed. *Anabolic Steroids in Sport and Exercise*. Champaign, IL: Human Kinetics; 51–71

The New England Journal of Medicine

© Copyright, 1996, by the Massachusetts Medical Society

VOLUME 335

JULY 4, 1996

NUMBER 1



THE EFFECTS OF SUPRAPHYSIOLOGIC DOSES OF TESTOSTERONE ON MUSCLE SIZE AND STRENGTH IN NORMAL MEN

SHALENDER BHASIN, M.D., THOMAS W. STORER, PH.D., NANCY BERMAN, PH.D., CARLOS CALLEGARI, M.D.,
BRENDA CLEVINGER, B.A., JEFFREY PHILLIPS, M.D., THOMAS J. BUNNELL, B.A., RAY TRICKER, PH.D., AIDA SHIRAZI, R.PH.,
AND RICHARD CASABURI, PH.D., M.D.

ABSTRACT

Background Athletes often take androgenic steroids in an attempt to increase their strength. The efficacy of these substances for this purpose is unsubstantiated, however.

Methods We randomly assigned 43 normal men to one of four groups: placebo with no exercise, testosterone with no exercise, placebo plus exercise, and testosterone plus exercise. The men received injections of 600 mg of testosterone enanthate or placebo weekly for 10 weeks. The men in the exercise groups performed standardized weight-lifting exercises three times weekly. Before and after the treatment period, fat-free mass was determined by underwater weighing, muscle size was measured by magnetic resonance imaging, and the strength of the arms and legs was assessed by bench-press and squatting exercises, respectively.

Results Among the men in the no-exercise groups, those given testosterone had greater increases than those given placebo in muscle size in their arms (mean [\pm SE] change in triceps area, 424 ± 104 vs. -81 ± 109 mm²; $P < 0.05$) and legs (change in quadriceps area, 607 ± 123 vs. -131 ± 111 mm²; $P < 0.05$) and greater increases in strength in the bench-press (9 ± 4 vs. -1 ± 1 kg, $P < 0.05$) and squatting exercises (16 ± 4 vs. 3 ± 1 kg, $P < 0.05$). The men assigned to testosterone and exercise had greater increases in fat-free mass (6.1 ± 0.6 kg) and muscle size (triceps area, 501 ± 104 mm²; quadriceps area, 1174 ± 91 mm²) than those assigned to either no-exercise group, and greater increases in muscle strength (bench-press strength, 22 ± 2 kg; squatting-exercise capacity, 38 ± 4 kg) than either no-exercise group. Neither mood nor behavior was altered in any group.

Conclusions Supraphysiologic doses of testosterone, especially when combined with strength training, increase fat-free mass and muscle size and strength in normal men. (N Engl J Med 1996;335:1-7.)

©1996, Massachusetts Medical Society.

ANABOLIC-ANDROGENIC steroids are widely abused by athletes and recreational bodybuilders because of the perception that these substances increase muscle mass and strength,¹⁻⁹ but this premise is unsubstantiated. Testosterone replacement increases nitrogen retention and fat-free mass in castrated animals and hypogonadal men,¹⁰⁻¹⁵ but whether supraphysiologic doses of testosterone or other anabolic-androgenic steroids augment muscle mass and strength in normal men is unknown.¹⁻⁹ Studies of the effects of such steroids on muscle strength have been inconclusive,¹⁶⁻³³ and several reviews have emphasized the shortcomings of the studies.^{1-5,8-10} Some of the studies were not randomized; most did not control for intake of energy and protein; the exercise stimulus was often not standardized; and some studies included competitive athletes whose motivation to win may have kept them from complying with a standardized regimen of diet and exercise.

We sought to determine whether supraphysiologic doses of testosterone, administered alone or in conjunction with a standardized program of strength-training exercise, increase fat-free mass and muscle size and strength in normal men. To overcome the pitfalls of previous studies, the intake of energy and protein and the exercise stimulus were standardized. Because some previous studies had demonstrated significant increases in muscle strength and hyper-

From the Department of Medicine, Charles R. Drew University of Medicine and Science, Los Angeles (S.B., C.C., B.C.); the Exercise Science Laboratory, El Camino College, Torrance, Calif. (T.W.S., T.J.B.); the Department of Medicine, Harbor-UCLA Medical Center, Torrance, Calif. (N.B., J.P., R.C.); and the Department of Public Health, Oregon State University, Corvallis (R.T., A.S.). Address reprint requests to Dr. Bhasin at the Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University of Medicine and Science, 1621 E. 120th St., MP #2, Los Angeles, CA 90059.

trophy in experienced athletes but not in sedentary subjects, we studied men who had weight-lifting experience.

METHODS

Study Design

This study was approved by the institutional review boards of the Harbor-UCLA Research and Education Institute and the Charles R. Drew University of Medicine and Science. All the study subjects gave informed written consent. The subjects were normal men weighing 90 to 115 percent of their ideal body weights; they were 19 to 40 years of age and had experience with weight lifting. They were recruited through advertisements in local newspapers and community colleges. None had participated in competitive sports in the preceding 12 months. Men who had ever taken anabolic agents or recreational drugs or had had a psychiatric or behavioral disorder were excluded from the study.

Of 50 men who were recruited, 7 dropped out during the control period because of problems with scheduling or compliance. The remaining 43 men were randomly assigned to one of four groups: placebo with no exercise, testosterone with no exercise, placebo plus exercise, and testosterone plus exercise. The study was divided into a 4-week control period, a 10-week treatment period, and a 16-week recovery period. During the four-week control period, the men were asked not to lift any weights or engage in strenuous aerobic exercise.

Of the 43 men, 3 dropped out during the treatment phase: 1 because of problems with compliance, 1 because illicit-drug use was detected by routine drug screening, and 1 because of an automobile accident. Forty men completed the study: 10 in the placebo, no-exercise group; 10 in the testosterone, no-exercise group; 9 in the placebo-plus-exercise group; and 11 in the testosterone-plus-exercise group.

Standardization of Protein and Energy Intake

Two weeks before day 1, the men were instructed to begin following a standardized daily diet containing 36 kcal per kilogram of body weight, 1.5 g of protein per kilogram, and 100 percent of the recommended daily allowance of vitamins, minerals, and trace elements. Compliance with the diet was verified every four weeks by three-day records of food consumption. The dietary intake was adjusted every two weeks on the basis of changes in body weight.

Treatment

The men received either 600 mg of testosterone enanthate in sesame oil or placebo intramuscularly each week for 10 weeks in the Clinical Research Center. This dose is six times higher than the dose usually given as replacement therapy in men with hypogonadism and is therefore supraphysiologic. Doses as high as 300 mg per week have been given to normal men for 16 to 24 weeks without major toxic effects.³⁴

Training Stimulus

The men in the exercise groups received controlled, supervised strength training three days per week during the treatment period. All the men trained at equivalent intensities in relation to their strength scores before the training. The training consisted of a cycle of weight lifting at heavy intensity (90 percent of the maximal weight the man lifted for one repetition before the start of training), light intensity (70 percent of the pretraining one-repetition maximal weight), and medium intensity (80 percent of this maximal weight) on three nonconsecutive days each week.³⁵ Regardless of the actual weights lifted, the training was held constant at four sets with six repetitions per set (a set is the number of complete repetitions of an exercise followed by rest). Because previous research had demonstrated increases in strength of ap-

proximately 7 percent for the bench-press exercise and 12 percent for the squatting exercise after four to five weeks of training,³⁵ the weights were increased correspondingly during the final five weeks of training in relation to the initial intensity. The number of sets was also increased from four to five, but the number of repetitions per set remained constant. The men were advised not to undertake any resistance exercise or moderate-to-heavy endurance exercise in addition to the prescribed regimen.

Evaluation and Outcome Measures

The primary end points were fat-free mass, muscle size as measured by magnetic resonance imaging (MRI), and muscle strength as based on the one-repetition maximal weight lifted during the bench-press and squatting exercises before and after the 10-week treatment period. Serum concentrations of total and free testosterone, luteinizing hormone, follicle-stimulating hormone, and sex hormone-binding globulin were measured on days 14 and 28 of the control period and days 2, 3, 7, 14, 28, 42, 56, and 70 of the treatment period. Blood counts, blood chemistry (including serum aminotransferases), serum concentrations of prostate-specific antigen, and plasma concentrations of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured at the start of the control period and on day 4; on days 28, 56, and 70 of the treatment period; and four months after the discontinuation of treatment. Periodic evaluations to identify adverse effects were performed by examiners unaware of the study-group assignments on days 1 and 28 of the control period; days 28, 56, and 70 of the treatment period; and four months after the discontinuation of treatment. Mood and behavior were evaluated during the first week of the control period and after 6 and 10 weeks of treatment. Sexual function and semen characteristics were not assessed.

Assessment of Muscle Size

Muscle size was measured by MRI of the arms and legs at the humeral or femoral mid-diaphyseal level, the junction of the upper third and middle third of the bone, and the junction of the middle third and lower third. The cross-sectional areas of the arms and legs, the subcutaneous tissue, the muscle compartment, and the quadriceps and triceps muscles were computed, and the areas at the three levels were averaged.

Analysis of Body Composition

Fat-free mass was estimated on the basis of measurements of body density obtained by underwater weighing. During weighing, the men were asked to exhale to the residual volume, as measured by helium dilution.

Measures of Muscle Strength

The effort-dependent performance of muscle was assessed on the basis of the maximal weight lifted for one repetition during the bench-press and squatting exercises.³⁶ Each man completed increasingly more difficult lifts with the same weights and bars that he used during training; in each exercise, the maximal weight lifted (the one-repetition maximum) was recorded as a measure of muscle strength.

Hormone Measurements

Serum concentrations of luteinizing hormone and follicle-stimulating hormone were measured by immunofluorometric assays,³⁶ each with a sensitivity of 0.05 IU per liter. Serum testosterone was measured by immunoassay,³⁷ and free testosterone was measured by equilibrium dialysis.³⁷ Serum concentrations of sex hormone-binding globulin and prostate-specific antigen were measured by immunoassays using reagents purchased from Delphia-Wallac (Turku, Finland) and Hybritech (San Diego, Calif.), respectively.

TABLE 3. SERUM CONCENTRATIONS OF ENDOCRINE HORMONES IN THE STUDY SUBJECTS BEFORE AND AFTER THE 10 WEEKS OF TREATMENT.*

| HORMONE | NO EXERCISE | | EXERCISE | |
|---------------------------------------|-------------|--------------|----------|--------------|
| | PLACEBO | TESTOSTERONE | PLACEBO | TESTOSTERONE |
| Total testosterone (ng/dl) | | | | |
| Base line | 516±58 | 502±63 | 557±45 | 431±38 |
| 10 wk | 453±35 | 2828±417†‡ | 667±117 | 3244±305†‡ |
| Free testosterone (pg/ml) | | | | |
| Base line | 74±7 | 79±7 | 83±7 | 90±6 |
| 10 wk | 74±13 | 497±62†‡ | 81±9 | 572±53†‡ |
| Luteinizing hormone (mIU/ml) | | | | |
| Base line | 3.3±0.4 | 3.8±0.6 | 4.0±0.7 | 3.3±0.5 |
| 10 wk | 4.3±0.9 | 0.4±0.2†‡ | 4.4±1.1 | 0.4±0.2†‡ |
| Follicle-stimulating hormone (mIU/ml) | | | | |
| Base line | 3.1±0.3 | 3.1±0.4 | 3.2±0.6 | 3.0±0.6 |
| 10 wk | 2.7±0.3 | 0.3±0.2†‡ | 4.4±1.1 | 0.10±0.03†‡ |
| Sex hormone-binding globulin (ng/dl) | | | | |
| Base line | 224±33 | 256±34 | 353±41 | 271±43 |
| 10 wk | 244±53 | 176±24‡§ | 320±31 | 201±34‡¶ |

*Values at 10 weeks were obtained 1 week after the final injection. To convert values for total testosterone to nanomoles per liter, multiply by 0.0347; to convert values for free testosterone to picomoles per liter, multiply by 3.47; to convert values for sex hormone-binding globulin to nanomoles per liter, multiply by 0.12. Plus-minus values are means ±SE.

†P<0.001 for the comparison with the corresponding base-line value.

‡P<0.05 for the comparison of the difference between this value and the base-line value with the corresponding difference in either placebo group.

§P=0.008 for the comparison with the corresponding base-line value.

¶P=0.05 for the comparison with the corresponding base-line value.

groups, but not in the placebo groups (Table 3). The base-line serum concentrations of luteinizing hormone, follicle-stimulating hormone, and sex hormone-binding globulin were similar in the four groups, and the concentrations decreased significantly in the two testosterone groups.

Body Weight and Composition

Body weight did not change significantly in the men in either placebo group (Table 4). The men given testosterone without exercise had a significant mean increase in total body weight, and those in the testosterone-plus-exercise group had an average increase of 6.1 kg in body weight — a greater increase than in the other three groups.

Fat-free mass did not change significantly in the group assigned to placebo but no exercise (Table 4 and Fig. 1). The men treated with testosterone but no exercise had an increase of 3.2 kg in fat-free mass, and those in the placebo-plus-exercise group had an increase of 1.9 kg. The increase in the testosterone-plus-exercise group was substantially greater (averaging 6.1 kg). The percentage of body fat did not change significantly in any group (data not shown).

Muscle Size

The mean cross-sectional areas of the arm and leg muscles did not change significantly in the placebo

groups, whether the men had exercise or not (Table 4 and Fig. 1). The men in the testosterone groups had significant increases in the cross-sectional areas of the triceps and the quadriceps (Table 4); the group assigned to testosterone without exercise had a significantly greater increase in the cross-sectional area of the quadriceps than the placebo-alone group, and the testosterone-plus-exercise group had greater increases in quadriceps and triceps area than either the testosterone-alone or the placebo-plus-exercise group (P<0.05).

Muscle Strength

Muscle strength in the bench-press and the squatting exercises did not change significantly over the 10-week period in the group assigned to placebo with no exercise. The men in the testosterone-alone and placebo-plus-exercise groups had significant increases in the one-repetition maximal weights lifted in the squatting exercises, averaging 19 percent and 21 percent, respectively (Table 4 and Fig. 1). Similarly, mean bench-press strength increased in these two groups by 10 percent and 11 percent, respectively. In the testosterone-plus-exercise group, the increase in muscle strength in the squatting exercise (38 percent) was greater than that in any other group, as was the increase in bench-press strength (22 percent).

TABLE 4. BODY WEIGHT, FAT-FREE MASS, AND MUSCLE SIZE AND STRENGTH BEFORE AND AFTER THE 10 WEEKS OF TREATMENT.*

| VARIABLE | NO EXERCISE | | EXERCISE | |
|------------------------------------|-------------|--------------|-------------|--------------|
| | PLACEBO | TESTOSTERONE | PLACEBO | TESTOSTERONE |
| Body weight (kg) | | | | |
| Base line | 79.5±4.3 | 82.2±1.9 | 85.5±3.3 | 76.0±3.0 |
| 10 wk | 80.8±4.4 | 85.7±1.5 | 86.4±2.9 | 82.0±2.8† |
| P value | — | 0.004 | — | <0.001 |
| Fat-free mass (kg) | | | | |
| Base line | 65.1±2.5 | 69.9±1.3 | 72.1±2.3 | 65.3±1.8 |
| 10 wk | 65.9±2.7 | 73.1±2.2 | 74.1±2.2 | 71.4±1.8‡ |
| P value | — | — | 0.017 | <0.001 |
| Triceps area (mm ²) | | | | |
| Base line | 3621±213 | 3579±260 | 4,052±262 | 3483±217 |
| 10 wk | 3539±226 | 4003±229§ | 4,109±230 | 3984±239§ |
| P value | — | 0.003 | — | <0.001 |
| Quadriceps area (mm ²) | | | | |
| Base line | 8796±561 | 9067±398 | 9,920±569 | 8550±353 |
| 10 wk | 8665±481 | 9674±472§ | 10,454±474§ | 9724±348¶ |
| P value | — | <0.001 | — | <0.001 |
| Bench-press exercise (kg lifted) | | | | |
| Base line | 88±5 | 96±8 | 109±12 | 97±6 |
| 10 wk | 88±5 | 105±8§ | 119±11§ | 119±6‡ |
| P value | — | — | 0.005 | <0.001 |
| Squatting exercise (kg lifted) | | | | |
| Base line | 102±6 | 103±8 | 126±13 | 102±5 |
| 10 wk | 105±6 | 116±5 | 151±13§ | 140±5¶ |
| P value | — | 0.004 | <0.001 | <0.001 |

*P values are shown for the comparison of the 10-week values with the base-line values when $P \leq 0.05$. Plus-minus values are means \pm SE.

† $P < 0.05$ for the comparison of the change from base line with that in either placebo group.

‡ $P < 0.05$ for the comparison of the change from base line with that in either no-exercise group.

§ $P < 0.05$ for the comparison of the change from base line with that in the group assigned to placebo with no exercise.

¶ $P < 0.05$ for the comparison of the change from base line with that in the other three groups.

Mood and Behavior

No differences were found between the exercise groups and the no-exercise groups or between the placebo groups and the testosterone groups in any of the five subcategories of anger assessed by the Multidimensional Anger Inventory. No significant changes in mood or behavior were reported by the men on the Mood Inventory or by their live-in partners, spouses, or parents on the Observer Mood Inventory.

DISCUSSION

Our results show that supraphysiologic doses of testosterone, especially when combined with strength training, increase fat-free mass, muscle size, and strength in normal men when potentially confounding variables, such as nutritional intake and exercise stimulus, are standardized. The combination of strength training and testosterone produced greater increases in muscle size and strength than were achieved with either intervention alone. The combined regimen of testosterone and exercise led to an increase of 6.1 kg in fat-free mass over the course of

10 weeks; this increase entirely accounted for the changes in body weight.

The exercise was standardized in all the men, and therefore the effects of testosterone on muscle size and strength cannot be attributed to more intense training in the groups receiving the treatment. Careful selection of experienced weight lifters, the exclusion of competitive athletes, and close follow-up ensured a high degree of compliance with the regimens of exercise, treatment, and diet, which was verified by three-day food records (data not shown) and the values obtained for serum testosterone, luteinizing hormone, and follicle-stimulating hormone. Except for one man who missed one injection, all the men received all their scheduled injections. It has been argued that studies in which large doses of androgens are used cannot be truly blinded because of the occurrence of acne or other side effects. In this study, neither the investigators nor the personnel performing the measurements knew the study-group assignments. Three men receiving testosterone and one man receiving placebo had acneiform eruptions; these men may have assumed themselves to be receiving testosterone. Thus, it cannot be stated with certainty

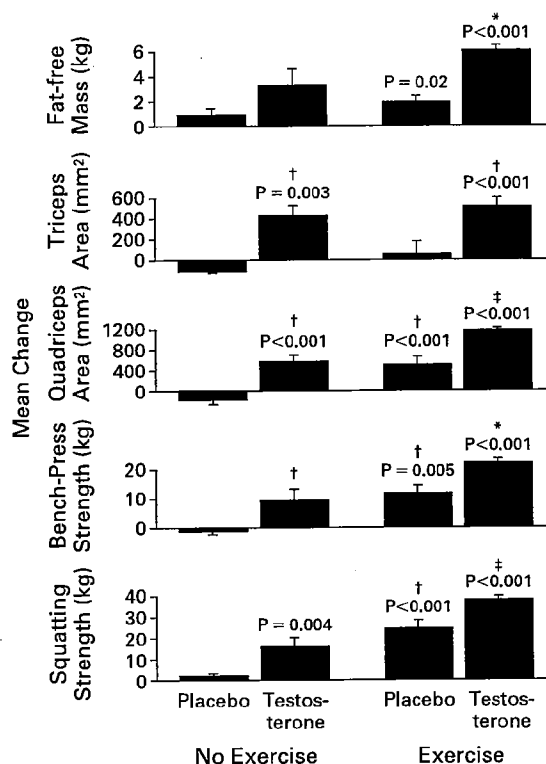


Figure 1. Changes from Base Line in Mean (\pm SE) Fat-free Mass, Triceps and Quadriceps Cross-Sectional Areas, and Muscle Strength in the Bench-Press and Squatting Exercises over the 10 Weeks of Treatment.

The P values shown are for the comparison between the change indicated and a change of zero. The asterisks indicate $P < 0.05$ for the comparison between the change indicated and that in either no-exercise group; the daggers, $P < 0.05$ for the comparison between the change indicated and that in the group assigned to placebo with no exercise; and the double daggers, $P < 0.05$ for the comparison between the change indicated and the changes in all three other groups.

that the men were completely unaware of the nature of their treatments.

The doses of androgenic steroids used in previous studies were low,^{1-5,11,12} mostly because of concern about potential toxic effects. In contrast, to our knowledge the dose of testosterone enanthate administered in this study (600 mg per week) is the highest administered in any study of athletic performance. Undoubtedly, some athletes and bodybuilders take even higher doses than those we gave. Furthermore, athletes often "stack" androgenic and anabolic steroids, taking multiple forms simultaneously. We do not know whether still higher doses of testosterone or the simultaneous administration of several steroids would have more pronounced effects. The absence of systemic toxicity during tes-

tosterone treatment was consistent with the results of studies of the contraceptive efficacy of that hormone.³⁴

The method used in this study to evaluate muscle performance on the basis of the one-repetition maximal weight lifted is dependent on effort. Although the men receiving testosterone did have increases in muscle size, some of the gains in strength may have resulted from the behavioral effects of testosterone.

The dose dependency of the action of testosterone on fat-free mass and protein synthesis has not been well studied. Forbes³⁹ proposed a single dose-response curve extending from the hypogonadal to the supraphysiologic range. Others have suggested that there may be two dose-response curves: one in the hypogonadal range, with maximal responses corresponding to the serum testosterone concentrations at the lower end of the range in normal men, and the second in the supraphysiologic range, presumably representing a separate mechanism of action—that is, a pathway of independent androgen receptors.^{1,40}

Supraphysiologic doses of testosterone, with or without exercise, did not increase the occurrence of angry behavior by these carefully selected men in the controlled setting of this experiment. Our results, however, do not preclude the possibility that still higher doses of multiple steroids may provoke angry behavior in men with preexisting psychiatric or behavioral problems.

Our results in no way justify the use of anabolic-androgenic steroids in sports, because, with extended use, such drugs have potentially serious adverse effects on the cardiovascular system, prostate, lipid metabolism, and insulin sensitivity. Moreover, the use of any performance-enhancing agent in sports raises serious ethical issues. Our findings do, however, raise the possibility that the short-term administration of androgens may have beneficial effects in immobilized patients, during space travel, and in patients with cancer-related cachexia, disease caused by the human immunodeficiency virus, or other chronic wasting disorders.

Supported by a grant (1 RO1 DK 45211) from the National Institutes of Health, by a General Clinical Research Center grant (MO-00543), and by grants (P20RR11145-01, a Clinical Research Infrastructure Initiative; and G12RR03026) from the Research Centers for Minority Institutions.

We are indebted to Dr. Indrani Sinha-Hikim for the serum hormone assays, to Dr. Paul Fu for the plasma lipid measurements, to the staff of the General Clinical Research Center for conducting the studies, and to BioTechnology General Corporation, Iselin, New Jersey, for providing testosterone enanthate.

REFERENCES

1. Wilson JD. Androgen abuse by athletes. *Endocr Rev* 1988;9:181-99.
2. Strauss RH, Yesalis CE. Anabolic steroids in the athlete. *Annu Rev Med* 1991;42:449-57.
3. Haupt HA, Rovere GD. Anabolic steroids: a review of the literature. *Am J Sports Med* 1984;12:469-84.
4. Cowart V. Steroids in sports: after four decades, time to return these genetics to bottle? *JAMA* 1987;257:421-3.
5. Elashoff JD, Jacknow AD, Shain SG, Braunstein GD. Effects of anabolic-androgenic steroids on muscular strength. *Ann Intern Med* 1991;115:387-93.
6. Wade N. Anabolic steroids: doctors denounce them, but athletes aren't listening. *Science* 1972;176:1399-403.
7. Buckley WE, Yesalis CE III, Friedl KE, Anderson WA, Streit AL, Wright JE. Estimated prevalence of anabolic steroid use among male high school seniors. *JAMA* 1988;260:3441-5.
8. Casaburi R, Storer T, Bhasin S. Androgen effects on body composition and muscle performance. In: Bhasin S, Gabelnick HL, Spieler JM, Swerdloff RS, Wang C, eds. *Pharmacology, biology, and clinical applications of androgens: current status and future prospects*. New York: Wiley-Liss, 1996:283-8.
9. American College of Sports Medicine. Position stand on the use of anabolic-androgenic steroids in sports. *Med Sci Sports Exerc* 1987;19:534-9.
10. Mooradian AD, Morley JE, Korenman SG. Biological actions of androgens. *Endocr Rev* 1987;8:1-28.
11. Kochakian CD. Comparison of protein anabolic property of various androgens in the castrated rat. *Am J Physiol* 1950;160:53-67.
12. Kenyon AT, Knowlton K, Sandiford I, Koch FC, Lotwin G. A comparative study of the metabolic effects of testosterone propionate in normal men and women and in eunuchoidism. *Endocrinology* 1940;26:26-45.
13. Bhasin S, Casaburi R, Berman N, et al. Replacement doses of testosterone increase lean body mass, muscle size and strength in hypogonadal men. Presented at the American Society of Andrology Meeting, Springfield, Ill., April 4-6, 1994. abstract.
14. Katznelson L, Finkelstein J, Baressi C, Klibanski A. Increase in trabecular bone density and altered body composition in androgen replaced hypogonadal men. In: Program and abstracts of the 76th Annual Meeting of the Endocrine Society, Anaheim, Calif., June 15-18, 1994. Bethesda, Md.: Endocrine Society Press, 1994:581. abstract.
15. Griggs RC, Kingston W, Jozefowicz RF, Herr BE, Forbes G, Halliday D. Effect of testosterone on muscle mass and muscle protein synthesis. *J Appl Physiol* 1989;66:498-503.
16. Ward P. The effect of an anabolic steroid on strength and lean body mass. *Med Sci Sports* 1973;5:277-82.
17. Johnson LC, Fisher G, Silvester LJ, Hofheins CC. Anabolic steroid: effects on strength, body weight, oxygen uptake, and spermatogenesis upon mature males. *Med Sci Sports* 1972;4:43-5.
18. Ariel G. The effect of anabolic steroid upon skeletal muscle contractile force. *J Sports Med Phys Fitness* 1973;13:187-90.
19. Hervey GR, Knibbs AV, Burkinshaw L, et al. Effects of methandienone on the performance and body composition of men undergoing athletic training. *Clin Sci* 1981;60:457-61.
20. Stamford BA, Moffatt R. Anabolic steroid: effectiveness as an ergogenic aid to experienced weight trainers. *J Sports Med Phys Fitness* 1974;14:191-7.
21. Win-May M, Mya-Tu M. The effect of anabolic steroids on physical fitness. *J Sports Med Phys Fitness* 1975;15:266-71.
22. Tahmindjis AJ. The use of anabolic steroids by athletes to increase body weight and strength. *Med J Aust* 1976;1:991-3.
23. Freed DLJ, Banks AJ, Longson D, Burley DM. Anabolic steroids in athletics: crossover double-blind trial on weightlifters. *BMJ* 1975;2:471-3.
24. Johnson LC, O'Shea JP. Anabolic steroid: effects on strength development. *Science* 1969;164:957-9.
25. Bowers RW, Reardon JP. Effects of methandrostenedione (Dianabol) on strength development and aerobic capacity. *Med Sci Sports* 1972;4:54. abstract.
26. Golding LA, Freyding JE, Fislief SS. Weight, size, and strength — unchanged with steroids. *Physician Sports Med* 1974;2(6):39-43.
27. Stromme SB, Meen HD, Aakvaag A. Effects of an androgenic-anabolic steroid on strength development and plasma testosterone levels in normal males. *Med Sci Sports* 1974;6:203-8.
28. Fahey TD, Brown CH. The effects of an anabolic steroid on the strength, body composition, and endurance of college males when accompanied by a weight training program. *Med Sci Sports* 1973;5:272-6.
29. Casner SW Jr, Early RG, Carlson BR. Anabolic steroid effects on body composition in normal young men. *J Sports Med Phys Fitness* 1971;11:98-103.
30. Samuels LT, Henschel AF, Keys A. Influence of methyl testosterone on muscular work and creatine metabolism in normal young men. *J Clin Endocrinol* 1942;2:649-54.
31. Loughton SJ, Ruhling RO. Human strength and endurance responses to anabolic steroid and training. *J Sports Med Phys Fitness* 1977;17:285-96.
32. Crist DM, Stackpole PJ, Peake GT. Effects of androgenic-anabolic steroids on neuromuscular power and body composition. *J Appl Physiol* 1983;54:366-70.
33. O'Shea JP, Winkler W. Biochemical and physical effects of an anabolic steroid in competitive swimmers and weightlifters. *Nutr Rep Int* 1970;2:351-62.
34. Matsumoto AM. Is high dosage testosterone an effective male contraceptive agent? *Fertil Steril* 1988;50:324-8.
35. Practical testing and progress evaluation. In: Stone MH, O'Bryen HS. *Weight training: a scientific approach*. Edina, Minn.: Burgess International Group, 1987.
36. Tom L, Bhasin S, Salameh W, et al. Male contraception: induction of azoospermia in normal men with combined gonadotropin-releasing hormone antagonist and testosterone enanthate. *J Clin Endocrinol Metab* 1992;75:476-83.
37. Bhasin S, Swerdloff RS, Steiner BS, et al. A biodegradable testosterone microcapsule formulation provides uniform eugonadal levels of testosterone for 10-11 weeks in hypogonadal men. *J Clin Endocrinol Metab* 1992;74:75-83.
38. Siegel JM. The Multidimensional Anger Inventory. *J Pers Soc Psychol* 1986;51:191-200.
39. Forbes GB. The effect of anabolic steroids on lean body mass: the dose response curve. *Metabolism* 1985;34:571-3.
40. Byerley LO, Lee WP, Buena F, et al. Effect of modulating serum testosterone in the normal male range on protein dynamics, carbohydrate and lipid metabolism. *Endocr J* 1993;1:253-9.

Testosterone dose-response relationships in healthy young men

SHALENDER BHASIN,¹ LINDA WOODHOUSE,¹ RICHARD CASABURI,³ ATAM B. SINGH,¹ DIMPLE BHASIN,³ NANCY BERMAN,³ XIANGHONG CHEN,⁴ KEVIN E. YARASHESKI,⁴ LYNNE MAGLIANO,² CONNIE DZEKOV,¹ JEANNE DZEKOV,¹ RACHELLE BROSS,³ JEFFREY PHILLIPS,³ INDRANI SINHA-HIKIM,¹ RUOQUING SHEN,¹ AND THOMAS W. STORER²

¹Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R. Drew University of Medicine and Science, Los Angeles 90059; ²Laboratory for Exercise Sciences, El Camino College, and ³Harbor-University of California Los Angeles Medical Center, Torrance, California 90502; and ⁴Biomedical Mass Spectrometric Research Resource, Department of Internal Medicine, Washington University, School of Medicine, St. Louis, Missouri 63110

Received 13 March 2001; accepted in final form 27 July 2001

Bhasin, Shalender, Linda Woodhouse, Richard Casaburi, Atam B. Singh, Dimple Bhasin, Nancy Berman, Xianghong Chen, Kevin E. Yarasheski, Lynne Magliano, Connie Dzekov, Jeanne Dzekov, Rachelle Bross, Jeffrey Phillips, Indrani Sinha-Hikim, Ruoqing Shen, and Thomas W. Storer. Testosterone dose-response relationships in healthy young men. *Am J Physiol Endocrinol Metab* 281: E1172–E1181, 2001.—Testosterone increases muscle mass and strength and regulates other physiological processes, but we do not know whether testosterone effects are dose dependent and whether dose requirements for maintaining various androgen-dependent processes are similar. To determine the effects of graded doses of testosterone on body composition, muscle size, strength, power, sexual and cognitive functions, prostate-specific antigen (PSA), plasma lipids, hemoglobin, and insulin-like growth factor I (IGF-I) levels, 61 eugonadal men, 18–35 yr, were randomized to one of five groups to receive monthly injections of a long-acting gonadotropin-releasing hormone (GnRH) agonist, to suppress endogenous testosterone secretion, and weekly injections of 25, 50, 125, 300, or 600 mg of testosterone enanthate for 20 wk. Energy and protein intakes were standardized. The administration of the GnRH agonist plus graded doses of testosterone resulted in mean nadir testosterone concentrations of 253, 306, 542, 1,345, and 2,370 ng/dl at the 25-, 50-, 125-, 300-, and 600-mg doses, respectively. Fat-free mass increased dose dependently in men receiving 125, 300, or 600 mg of testosterone weekly (change +3.4, 5.2, and 7.9 kg, respectively). The changes in fat-free mass were highly dependent on testosterone dose ($P = 0.0001$) and correlated with log testosterone concentrations ($r = 0.73$, $P = 0.0001$). Changes in leg press strength, leg power, thigh and quadriceps muscle volumes, hemoglobin, and IGF-I were positively correlated with testosterone concentrations, whereas changes in fat mass and plasma high-density lipoprotein (HDL) cholesterol were negatively correlated. Sexual function, visual-spatial cognition and mood, and PSA levels did not change significantly at any

dose. We conclude that changes in circulating testosterone concentrations, induced by GnRH agonist and testosterone administration, are associated with testosterone dose- and concentration-dependent changes in fat-free mass, muscle size, strength and power, fat mass, hemoglobin, HDL cholesterol, and IGF-I levels, in conformity with a single linear dose-response relationship. However, different androgen-dependent processes have different testosterone dose-response relationships.

sexual function; testosterone effects on muscle; cognitive function; plasma lipids; prostate-specific antigen; testosterone effects on insulin-like growth factor I; testosterone and hemoglobin

TESTOSTERONE regulates many physiological processes, including muscle protein metabolism, some aspects of sexual and cognitive functions, secondary sex characteristics, erythropoiesis, plasma lipids, and bone metabolism (7, 50). However, testosterone dose dependency of various androgen-dependent processes is not well understood (6). Administration of replacement doses of testosterone to hypogonadal men (10, 12, 30, 45, 49) and of supraphysiological doses to eugonadal men (9, 22–23, 26) increases fat-free mass, muscle size, and strength. Conversely, suppression of endogenous testosterone concentrations is associated with loss of fat-free mass and a decrease in fractional muscle protein synthesis (33). However, not known are whether testosterone effects on the muscle are dose dependent, or the nature of the testosterone dose-response relationships (6). Androgen receptors in most tissues are either saturated or downregulated at physiological testosterone concentrations (2, 18, 39, 50); this leads to speculation that there might be two separate dose-response curves: one in hypogonadal range, with

Address for reprint requests and other correspondence: S. Bhasin, Professor of Medicine, UCLA School of Medicine, and Chief, Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R. Drew Univ. of Medicine and Science, 1731 E. 120th St., Los Angeles, CA 90059 (E-mail: SBHASIN@UCLA.EDU).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

maximal response at low normal testosterone concentrations, and a second in supraphysiological range, representing a separate mechanism of action (1). However, testosterone dose-response relationships for a range of androgen-dependent functions in humans have not been studied.

Animal studies suggest that different androgen-dependent processes have different androgen dose-response relationships (6, 8, 21). Sexual function in male mammals is maintained at serum testosterone concentrations that are at the lower end of the male range (3, 6, 8, 13, 21, 31). However, it is not known whether the low normal testosterone levels that normalize sexual function are sufficient to maintain muscle mass and strength, or whether the higher testosterone concentrations required to maintain muscle mass and strength might adversely affect plasma lipids, hemoglobin levels, and the prostate. This information is important for optimizing testosterone replacement regimens for treatment of hypogonadal men. Also, for the proposed use of testosterone in sarcopenia associated with aging (46, 47) and chronic illness (11, 27), it is important to know whether significant gains in muscle mass and strength can be achieved at testosterone doses that do not adversely affect plasma high-density lipoprotein (HDL) and prostate-specific antigen (PSA) levels.

Therefore, the primary objective of this study was to determine the dose dependency of testosterone's effects on fat-free mass and muscle performance. We hypothesized that changes in circulating testosterone concentrations would be associated with dose-dependent changes in fat-free mass, muscle strength, and power in conformity with a single linear dose-response relationship, and that the dose requirements for maintaining other androgen-dependent processes would be different. We treated young men with a long-acting gonadotropin-releasing hormone (GnRH) agonist to suppress endogenous testosterone secretion, and concomitantly also with one of five testosterone-dose regimens to create different levels of serum testosterone concentrations extending from subphysiological to the supraphysiological range. The lowest testosterone dose, 25 mg weekly, was selected because this dose had been shown to maintain sexual function in GnRH antagonist-treated men (37). The selection of the 600-mg weekly dose was based on the consideration that this was the highest dose that had been safely administered to men in controlled studies (9).

METHODS

This was a double-blind, randomized study consisting of a 4-wk control period, a 20-wk treatment period, and a 16-wk recovery period. Each participant provided informed consent, approved by the institutional review boards of Drew University and Harbor-UCLA Research and Education Institute.

Participants. The participants were healthy men, 18–35 yr of age, with prior weight-lifting experience and normal testosterone levels. These men had not used any anabolic agents and had not participated in competitive sports events in the

preceding year, and they were not planning to participate in competitive events in the following year.

Randomization. Sixty-one eligible men were randomly assigned to one of five groups. All received monthly injections of a long-acting GnRH agonist to suppress endogenous testosterone production. In addition, *group 1* received 25 mg of testosterone enanthate intramuscularly weekly; *group 2*, 50 mg testosterone enanthate; *group 3*, 125 mg testosterone enanthate; *group 4*, 300 mg testosterone enanthate; and *group 5*, 600 mg testosterone enanthate. Twelve men were assigned to *group 1*, 12 to *group 2*, 12 to *group 3*, 12 to *group 4*, and 13 to *group 5*. Testosterone and GnRH agonist injections were administered by the General Clinical Research Center staff to assure compliance.

Nutritional intake. Energy and protein intakes were standardized at 36 kcal·kg⁻¹·day⁻¹ and 1.2 g·kg⁻¹·day⁻¹, respectively. The standardized diet was initiated 2 wk before treatment was started; dietary instructions were reinforced every 4 wk. The nutritional intake was verified by analysis of 3-day food records and 24-h food recalls every 4 wk by use of the Minnesota Nutritional Software.

Exercise stimulus. The participants were asked not to undertake strength training or moderate-to-heavy endurance exercise during the study. These instructions were reinforced every 4 wk.

Outcome measures. Body composition and muscle performance were assessed at baseline and during *week 20*. Fat-free mass and fat mass were measured by underwater weighing and dual-energy X-ray absorptiometry (DEXA, Hologic 4500, Waltham, MA). Total thigh muscle and quadriceps muscle volumes were measured by MRI scanning.

For estimation of total body water, the men ingested 10 g of ²H₂O (10, 11), and plasma samples were drawn at -15, 0, 120, 180, and 240 min. We measured ²H abundance in plasma by nuclear magnetic resonance spectroscopy (10, 11), with a correction factor of 0.985 for exchangeable hydrogen. We measured bilateral leg press strength by use of the one-repetition maximum (1-RM) method (11). A seated leg press exercise with pneumatic resistance (Keiser Sport, Fresno, CA) was used for this purpose. Subjects performed 5–10 min of leg cycling and stretching warm-up and received instruction and practice in lifting mechanics before performing progressive warm-up lifts leading to the 1-RM. Seat position and the ensuing knee and hip angles, as well as foot placement, were measured and recorded for use in subsequent testing. To ensure reliability in this highly effort-dependent test, the 1-RM score was reassessed within 7 days, but not sooner than 2 days, after the first evaluation. If duplicate scores were within 5%, the higher of the two values was accepted as the strength score. If the two tests differed by >5%, additional studies were conducted, ≥2 days apart but within 7 days, until the two highest scores were within 5%. No subject required >2 days of strength assessment.

We also measured leg power, because power in the lower extremity is strongly related to performance of functional activities in the elderly (4). The sarcopenia that accompanies aging is due in large part to a loss of the fast-twitch type II fibers and the coincident decrease in explosive force. Muscular power is important in performing such daily activities as rising from a chair, climbing stairs, and walking with speed (4). Leg power was measured with a previously validated (4, 5) Nottingham leg extensor power rig. Subjects performed 10–15 trials of right leg and hip extension, attempting to generate as much force as possible by accelerating the leg rig's weighted flywheel from rest. The power score (in watts) was taken as the highest value observed during these trials with evidence of a plateau. As with the strength tests, power

measurements were preceded by a 5- to 10-min warm-up, stretching, and practice. The power tests were repeated within 7 days, but not sooner than 2 days, after the first tests to reduce the effect of familiarization. To minimize test-retest variability, the angle of knee flexion and the seat position were recorded and maintained constant across tests.

Sexual function was assessed by daily logs of sexual activity and desire that were maintained for 7 consecutive days at baseline and during treatment by use of a published instrument (13). Spatial cognition was assessed by a computerized checkerboard test (38) and mood by Hamilton's depression (20) and Young's mania scales (24).

Adverse experiences, blood counts and chemistries, PSA, plasma lipids, total and free testosterone, luteinizing hormone (LH), sex steroid-binding globulin (SHBG), and insulin-like growth factor I (IGF-I) levels were measured periodically during control and treatment periods. Serum total testosterone was measured by an immunoassay (8–11); free testosterone by equilibrium dialysis (43); LH, SHBG, and PSA by immunoradiometric assays (9–11); and IGF-I by acid-ethanol extraction and immunoassay (28). The sensitivities and intra- and interassay coefficients of variation for hormone assays were as follows: total testosterone (0.6 ng/dl), 8.2 and 13.2%; free testosterone (0.22 pg/ml), 4.2 and 12.3%; LH (0.05 U/l), 10.7 and 13.0%; SHBG (6.25 nmol/l), 4 and 6%; PSA (0.01 ng/ml), 5.0 and 6.4%; and IGF-I (80 ng/ml), 4 and 6%, respectively. These assays have been validated previously (8–11).

Statistical analyses. All variables were examined for their distribution characteristics. Variables not meeting the assumption of a normal distribution were log-transformed and retested. An ANOVA was used to compare change from baseline in outcome measures among the five groups. All outcome measures were analyzed using a paired *t*-test to detect a nonzero change from baseline within each group. $P < 0.05$ was considered statistically significant.

To describe the relationship between testosterone concentrations (T) and change in fat-free mass (Y) during testosterone administration, we tested three models: a linear model ($Y = a + bT$); a logarithmic model, $Y = a + b \cdot X$, where $X = \log(T)$, and a and b represent the intercept and slope, respectively; and a growth model, $Y = a/(1 + b \cdot e^{-k \cdot X})$. The logarithmic model provided the best fit for the data and was used to model the effects of testosterone concentrations on the change in other outcome variables. The correlations between testosterone concentrations and change in outcome variables are derived from this model. We also modeled the linear dependence of the change in outcome variables on testosterone dose by use of linear regression.

RESULTS

Participant characteristics. Of 61 men enrolled, 54 completed the study: 12 in group 1, 8 in group 2, 11 in group 3, 10 in group 4, and 13 in group 5. One man discontinued treatment because of acne; other subjects were unable to meet the demands of the protocol. The five groups did not significantly differ with respect to their baseline characteristics (Table 1).

Compliance. All evaluable subjects received 100% of their GnRH agonist injections, and only one man in the 125-mg group missed one testosterone injection.

Nutritional intake. Daily energy intake and proportion of calories derived from protein, carbohydrate, and fat were not significantly different among the five groups at baseline. There was no significant change in daily caloric, protein, carbohydrate, or fat intake in any group during treatment (data not shown).

Hormone levels. Serum total and free testosterone levels (Table 2), measured during week 16, 1 wk after the previous injection, were linearly dependent on the testosterone dose ($P = 0.0001$). Serum total and free testosterone concentrations decreased from baseline in men receiving the 25- and 50-mg doses and increased at 300- and 600-mg doses. Serum LH levels were suppressed in all groups. Serum SHBG levels decreased dose dependently at the 300- and 600-mg doses but did not change in other groups. Serum IGF-I concentrations increased dose dependently at the 300- and 600-mg doses (correlation between log testosterone level and change in IGF-I = 0.55, $P = 0.0001$). IGFBP-3 levels did not change significantly in any group.

Body composition. Fat-free mass, measured by underwater weighing, did not change significantly in men receiving the 25- or 50-mg testosterone dose, but it increased dose dependently at higher doses (Table 3). The changes in fat-free mass were highly dependent on testosterone dose ($P = 0.0001$) and correlated with log total testosterone concentrations during treatment ($r = 0.73$, $P = 0.0001$, see Fig. 2).

Changes in fat-free mass, measured by DEXA scan, were qualitatively similar to those obtained from underwater weighing (Table 3, Fig. 1). The measurements of fat-free mass by DEXA were highly correlated with values obtained from underwater weighing ($r = 0.94$, $P < 0.0001$).

Table 1. Baseline characteristics of the participants

| GnRH Agonist Testosterone Enanthate | + | + | + | + | + | P Value |
|--|---------------|--------------|--------------|--------------|--------------|---------|
| | 25 mg | 50 mg | 125 mg | 300 mg | 600 mg | |
| Age, yr | 28 ± 5 | 29 ± 5 | 28 ± 3 | 24 ± 5 | 25 ± 4 | 0.0583 |
| Height, cm | 175 ± 5 | 177 ± 9 | 178 ± 7 | 177 ± 7 | 175 ± 8 | 0.7230 |
| Weight, kg | 68.0 ± 8.4 | 77.0 ± 8.1 | 78.9 ± 10.6 | 78.4 ± 10.1 | 74.8 ± 12.5 | 0.1014 |
| Body mass index, kg/m ² | 23 ± 3 | 25 ± 3 | 25 ± 3 | 25 ± 3 | 25 ± 3 | 0.3680 |
| Serum testosterone levels, nmol/l | 593 ± 161 | 566 ± 220 | 553 ± 182 | 654 ± 157 | 632 ± 228 | 0.7093 |
| Fat-free mass, kg | 59.1 ± 6.7 | 65.1 ± 5.1 | 66.0 ± 7.2 | 67.3 ± 8.9 | 64.2 ± 8.0 | 0.1506 |
| Leg press strength, kg | 355.5 ± 103.8 | 407.8 ± 62.2 | 419.2 ± 86.2 | 439.8 ± 81.4 | 431.6 ± 99.3 | 0.2149 |
| Hemoglobin, g/l | 144 ± 12 | 151 ± 11 | 142 ± 9 | 144 ± 8 | 141 ± 8 | 0.1428 |
| No. in group | 12 | 12 | 12 | 12 | 13 | |

Values are means ± SD. GnRH, gonadotropin-releasing hormone.

Table 2. Serum total and free testosterone, LH, FSH, SHBG, and IGF-I levels

| Testosterone Dose | Baseline | Week 16 | Change from Baseline | P vs. Zero Change |
|---|------------|-------------|----------------------|-------------------|
| <i>Testosterone (ng/dl) (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 593 ± 48 | 253 ± 66 | -340 ± 85 | 0.0029 |
| 50 mg | 566 ± 78 | 306 ± 58 | -260 ± 64 | 0.0037 |
| 125 mg | 553 ± 53 | 570 ± 75 | 57 ± 75 | 0.7425 |
| 300 mg | 653 ± 50 | 1,345 ± 139 | 691 ± 143 | 0.0005 |
| 600 mg | 632 ± 63 | 2,370 ± 150 | 1,737 ± 156 | 0.0001 |
| <i>Free testosterone (pg/ml) (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 62 ± 6 | 29 ± 5 | -33 ± 8 | 0.0014 |
| 50 mg | 57 ± 6 | 32 ± 3 | -25 ± 5 | 0.0009 |
| 125 mg | 49 ± 5 | 52 ± 8 | 3 ± 7 | 0.8601 |
| 300 mg | 71 ± 7 | 138 ± 21 | 67 ± 18 | 0.0012 |
| 600 mg | 64 ± 5 | 275 ± 30 | 211 ± 31 | 0.0001 |
| <i>LH (U/l) (overall ANOVA P = 0.8054)</i> | | | | |
| 25 mg | 3.5 ± 0.4 | 0.3 ± 0.1 | -3.2 ± 0.4 | 0.0001 |
| 50 mg | 3.8 ± 0.3 | 0.6 ± 0.3 | -3.0 ± 0.4 | 0.0008 |
| 125 mg | 3.4 ± 0.3 | 0.5 ± 0.1 | -2.8 ± 0.4 | 0.0001 |
| 300 mg | 3.7 ± 0.5 | 0.6 ± 0.1 | -3.5 ± 0.5 | 0.0002 |
| 600 mg | 3.3 ± 0.3 | 0.6 ± 0.4 | -2.9 ± 0.4 | 0.0001 |
| <i>SHBG (nmol/l) (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 29.1 ± 2.9 | 28.5 ± 3.6 | -0.6 ± 2.9 | 0.8497 |
| 50 mg | 24.4 ± 3.4 | 21.1 ± 3.2 | -3.3 ± 1.1 | 0.0202 |
| 125 mg | 33.1 ± 4.2 | 28.9 ± 3.8 | -4.2 ± 2.6 | 0.1410 |
| 300 mg | 31.4 ± 3.8 | 22.4 ± 3.9 | -9.1 ± 3.7 | 0.0348 |
| 600 mg | 40.1 ± 4.9 | 20.6 ± 3.2 | -19.5 ± 2.8 | 0.0001 |
| <i>IGF-I (ng/ml) (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 268 ± 26 | 261 ± 35 | -7 ± 19 | 0.7462 |
| 50 mg | 246 ± 14 | 225 ± 12 | -20 ± 10 | 0.0797 |
| 125 mg | 299 ± 24 | 282 ± 31 | -18 ± 17 | 0.3284 |
| 300 mg | 314 ± 24 | 388 ± 30 | 74 ± 28 | 0.0272 |
| 600 mg | 227 ± 20 | 304 ± 21 | 77 ± 13 | 0.0001 |

Values on each day represent the mean (±SE) of all available values on that day. However, the change represents the difference between paired values only. Treatment values represent the day 113 (week 16) values, obtained 1 wk after the previous testosterone injection. We used week 16 rather than week 20 values because week 20 values were not always drawn exactly 1 wk after the previous injection. LH and FSH, luteinizing and follicle-stimulating hormones, respectively; SHBG, sex hormone-binding globulin; IGF-I, insulin-like growth factor I. To convert total testosterone levels to nmol/l, multiply by 0.03467. To convert free testosterone levels to pg/ml, multiply by 3.467.

To determine whether the apparent changes in fat-free mass by DEXA scan and underwater weighing represented water retention, we measured total body water and compared the ratios of total body water to fat-free mass before and after treatment in each group. The ratios of total body water to fat-free mass by underwater weighing did not significantly change with treatment in any treatment group (Table 3), indicating that the apparent increase in fat-free mass measured by underwater weighing did not represent water retention in excess of that associated with protein accretion.

Fat mass, measured by underwater weighing, increased significantly in men receiving the 25- and 50-mg doses but did not change in men receiving the higher doses of testosterone (Table 3, Fig. 1). There was an inverse correlation between change in fat mass

by underwater weighing and log testosterone concentrations ($r = -0.60$, $P = 0.0001$, Fig. 2).

Muscle size. The thigh muscle volume and quadriceps muscle volume did not significantly change in men receiving the 25- or 50-mg doses but increased dose dependently at higher doses of testosterone (Table 4, Fig. 1). The changes in thigh muscle and quadriceps muscle volumes correlated with log testosterone levels during treatment ($r = 0.66$, $P = 0.0001$, and $r = 0.55$, $P = 0.0001$, respectively, Fig. 2).

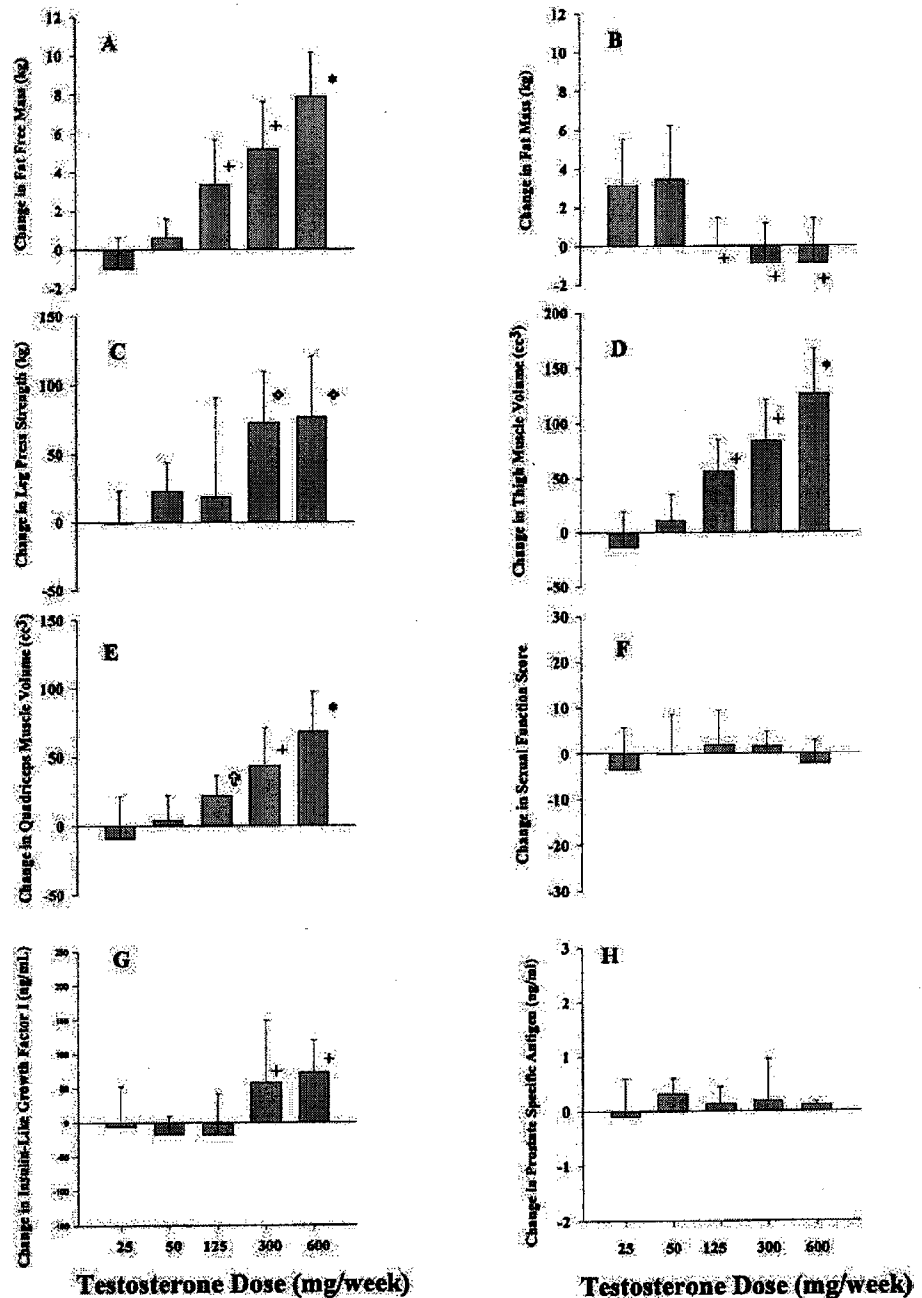
Muscle performance. The leg press strength did not change significantly in the 25- and 125-mg-dose groups but increased significantly in those receiving the 50-, 300-, and 600-mg doses (Table 5). The changes in leg press strength correlated with log testosterone levels during treatment ($r = 0.48$, $P = 0.0005$, Fig. 2) and changes in muscle volume ($r = 0.54$, $P = 0.003$) and fat-free mass ($r = 0.74$, $P < 0.0001$).

Table 3. Body composition analysis

| Testosterone Dose | Baseline | Week 20 | Change from Baseline | P vs. Zero Change |
|---|------------|-------------|----------------------|-------------------|
| <i>Fat-free mass (kg) by underwater weighing (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 61.1 ± 2.7 | 58.1 ± 1.7 | -1.0 ± 0.5 | 0.0695 |
| 50 mg | 66.1 ± 2.5 | 65.7 ± 2.0 | +0.6 ± 0.4 | 0.1324 |
| 125 mg | 66.0 ± 2.1 | 67.9 ± 2.7 | +3.4 ± 0.8 | 0.0024 |
| 300 mg | 66.9 ± 2.4 | 72.4 ± 2.8 | +5.2 ± 0.8 | 0.0001 |
| 600 mg | 64.2 ± 2.2 | 72.1 ± 2.4 | +7.9 ± 0.6 | 0.0001 |
| <i>Fat mass (kg) by underwater weighing (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 8.3 ± 1.4 | 11.3 ± 1.6 | +3.1 ± 0.7 | 0.0014 |
| 50 mg | 10.9 ± 1.4 | 14.3 ± 1.7 | +3.5 ± 1.0 | 0.0096 |
| 125 mg | 12.2 ± 2.0 | 10.9 ± 2.1 | +0.01 ± 0.5 | 0.9820 |
| 300 mg | 11.4 ± 1.6 | 10.9 ± 1.7 | -0.5 ± 0.6 | 0.4134 |
| 600 mg | 9.4 ± 1.9 | 8.8 ± 1.9 | -1.1 ± 0.7 | 0.1132 |
| <i>Fat-free mass (kg) by DEXA scan (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 53.6 ± 1.8 | 53.4 ± 2.0 | +0.4 ± 0.3 | 0.2198 |
| 50 mg | 58.6 ± 2.3 | 59.2 ± 2.5 | +1.1 ± 0.9 | 0.2313 |
| 125 mg | 60.1 ± 2.1 | 63.1 ± 2.3 | +2.9 ± 0.8 | 0.0054 |
| 300 mg | 59.0 ± 2.7 | 64.3 ± 2.2 | +5.5 ± 0.7 | 0.0001 |
| 600 mg | 57.4 ± 1.9 | 66.3 ± 2.4 | +8.9 ± 0.8 | 0.0001 |
| <i>Fat mass (kg) by DEXA scan (overall ANOVA P = 0.0004)</i> | | | | |
| 25 mg | 10.0 ± 1.8 | 13.7 ± 1.4 | +3.6 ± 1.5 | 0.0326 |
| 50 mg | 15.4 ± 1.2 | 17.9 ± 1.2 | +2.6 ± 1.0 | 0.0324 |
| 125 mg | 15.2 ± 2.0 | 15.2 ± 1.9 | -0.3 ± 0.8 | 0.6882 |
| 300 mg | 16.3 ± 1.2 | 15.41 ± 1.5 | -0.9 ± 0.6 | 0.1834 |
| 600 mg | 14.2 ± 1.9 | 12.0 ± 1.5 | -2.0 ± 0.7 | 0.0141 |
| <i>Ratio of total body water to fat-free mass (percent) (overall ANOVA for change from baseline, P = 0.270)</i> | | | | |
| 25 mg | 62.7 ± 2.7 | 63.7 ± 2.1 | +1.1 ± 2.4 | |
| 50 mg | 62.0 ± 1.9 | 63.8 ± 2.4 | +2.0 ± 2.0 | |
| 125 mg | 67.0 ± 1.7 | 63.5 ± 3.0 | -3.8 ± 1.6 | |
| 300 mg | 61.6 ± 2.7 | 64.6 ± 3.1 | +2.1 ± 2.5 | |
| 600 mg | 65.3 ± 2.4 | 67.4 ± 2.8 | +2.5 ± 1.7 | |

Values on each day represent the mean (±SE) of all available values on that day. However, the change represents the difference between paired values only. Ratios of total body water assessed by deuterium water dilution to fat-free mass measured by underwater weighing were calculated for each subject and averaged across subjects within each group. DEXA, dual-energy X-ray absorptiometry.

Fig. 1. Change in fat-free mass (A), fat mass (B), leg press strength (C), thigh muscle volume (D), quadriceps muscle volume (E), sexual function (F), insulin-like growth factor I (G), and prostate-specific antigen (H). Data are means \pm SE. *Significant differences from all other groups ($P < 0.05$); \diamond significant difference from 25-, 50-, and 125-mg doses ($P < 0.05$); + significant difference from 25- and 50-mg doses ($P < 0.05$); and \dagger significant difference from 25-mg dose ($P < 0.05$).



Leg power, measured by the Nottingham leg rig, did not change significantly in men receiving the 25-, 50-, and 125-mg doses of testosterone weekly, but it increased significantly in those receiving the 300- and 600-mg doses. The increase in leg power correlated with log testosterone concentrations ($r = 0.39$, $P = 0.0105$, Fig. 2) and changes in fat-free mass ($r = 0.30$, $P = 0.0392$) and muscle strength ($r = 0.42$, $P = 0.0020$).

Behavioral measures. The scores for sexual activity and sexual desire measured by daily logs did not change significantly at any dose. Similarly, visual-

spatial cognition (Table 6) and mood, as assessed by Hamilton's depression and Young's manic scales (data not shown), did not change significantly in any group.

Adverse experiences and safety measures. Hemoglobin levels decreased significantly in men receiving the 50-mg dose but increased at the 600-mg dose; the changes in hemoglobin were positively correlated with testosterone concentrations ($r = 0.66$, $P = 0.0001$) (Table 7). Changes in plasma HDL cholesterol, in contrast, were negatively dependent on testosterone dose ($P = 0.0049$) and correlated with testosterone concentrations ($r =$

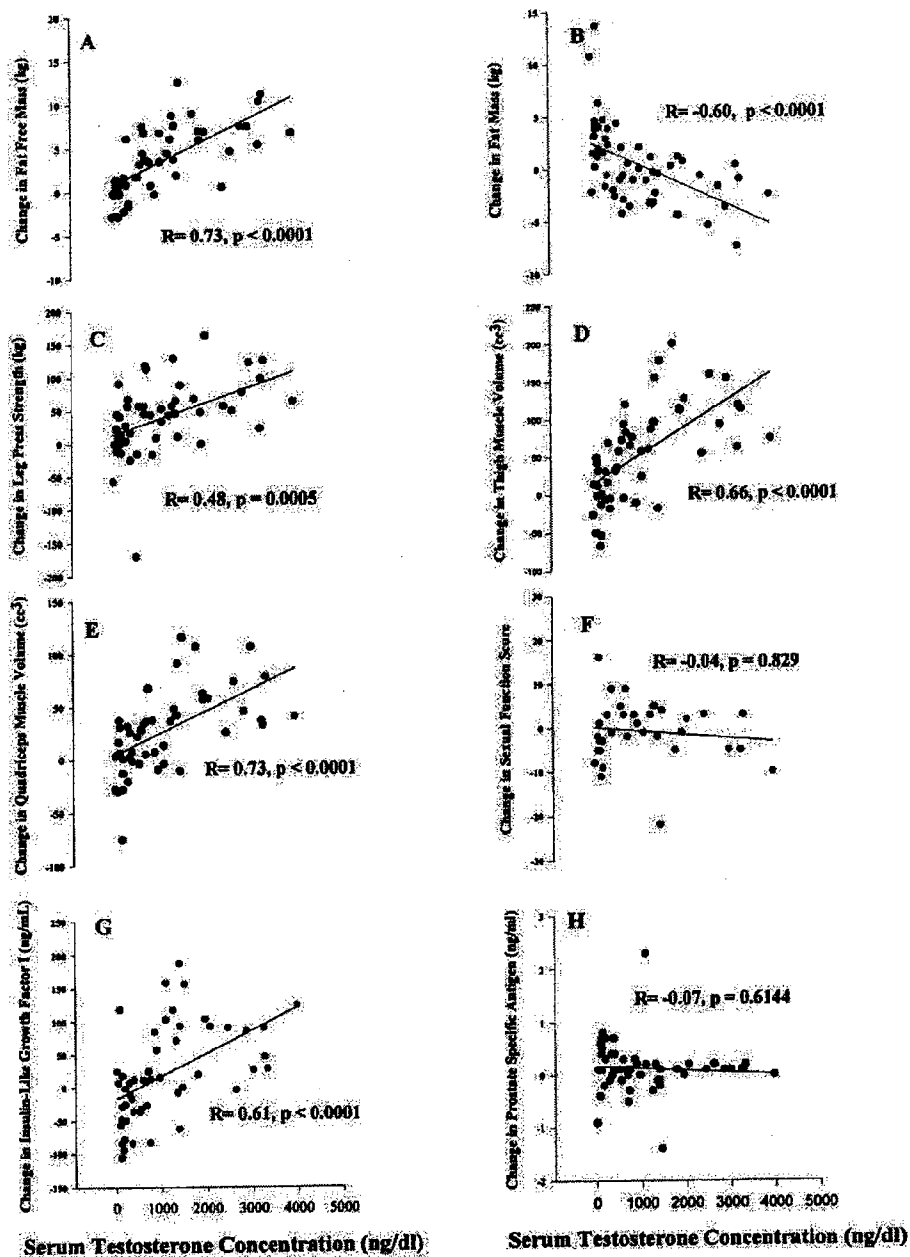


Fig. 2. Relationship between serum testosterone concentrations (T) during treatment (week 16) and change in fat-free mass (A), fat mass (B), leg press strength (C), thigh muscle volume (D), quadriceps muscle volume (E), sexual function (F), insulin-like growth factor I (G), and prostate-specific antigen (H). The correlation coefficient, r , was calculated using the logarithmic model, $Y = a + b \cdot X$, where $X = \log(T)$, and a and b represent the intercept and slope.

$-0.40, P = 0.0054$). Total cholesterol, plasma low-density lipoprotein cholesterol, and triglyceride levels did not change significantly at any dose. Serum PSA, creatinine, bilirubin, alanine aminotransferase, and alkaline phosphatase did not change significantly in any group, but aspartate aminotransferase decreased significantly in the 25-mg group. Two men in the 25-mg group, five in the 50-mg group, three in the 125-mg group, seven in the 300-mg group, and two in the 600-mg group developed acne. One man receiving the 50-mg dose reported decreased ability to achieve erections.

DISCUSSION

GnRH agonist administration suppressed endogenous LH and testosterone secretion; therefore, circulating testosterone concentrations during treatment were proportional to the administered dose of testosterone enanthate. This strategy of combined administration of GnRH agonist and graded doses of testosterone enanthate was effective in establishing different levels of serum testosterone concentrations among the five treatment groups. The different levels of circulating testosterone concentrations created by this regimen were associated with dose- and concentration-

Table 4. *Thigh and quadriceps muscle volume measured by MRI*

| Testosterone Dose | Baseline | Week 20 | Change from Baseline | P vs. zero change |
|--|----------|----------|----------------------|-------------------|
| <i>Thigh muscle volume (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 753 ± 46 | 739 ± 44 | -14 ± 10 | 0.1958 |
| 50 mg | 833 ± 53 | 844 ± 58 | 11 ± 8 | 0.2332 |
| 125 mg | 890 ± 49 | 966 ± 60 | 56 ± 10 | 0.0004 |
| 300 mg | 849 ± 39 | 933 ± 39 | 84 ± 12 | 0.0001 |
| 600 mg | 802 ± 45 | 928 ± 48 | 126 ± 12 | 0.0001 |
| <i>Quadriceps muscle volume (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 436 ± 30 | 427 ± 27 | -9 ± 9 | 0.3524 |
| 50 mg | 489 ± 34 | 493 ± 36 | 4 ± 7 | 0.5889 |
| 125 mg | 508 ± 29 | 546 ± 36 | 21 ± 5 | 0.0027 |
| 300 mg | 497 ± 25 | 540 ± 22 | 43 ± 9 | 0.0008 |
| 600 mg | 472 ± 27 | 540 ± 31 | 68 ± 8 | 0.0001 |

Values (in cm³) on each day represent the mean (±SE) of all available values on that day. However, the change represents the difference between paired values only.

dependent changes in fat-free mass, fat mass, thigh and quadriceps muscle volume, muscle strength, leg power, hemoglobin, circulating IGF-I, and plasma HDL cholesterol. Serum PSA levels, sexual desire and activity, and spatial cognition did not change significantly at any dose. The changes in fat-free mass, muscle volume, leg press strength and power, hemoglobin, and IGF-I were positively correlated, whereas changes in plasma HDL cholesterol and fat mass were negatively correlated with testosterone dose and total and free testosterone concentrations during treatment.

The compliance with the treatment regimen was high. The participants received 100% of their scheduled GnRH agonist, and 99% of testosterone injections. Serum LH levels were suppressed in all men, demonstrating the effectiveness of GnRH agonist treatment. The treatment regimen was well tolerated. There were no significant changes in PSA or liver enzymes at any dose. However, long-term effects of androgen administration on the prostate, cardiovascular risk, and behavior are unknown.

Table 5. *Change in measures of muscle performance*

| Testosterone Dose | Baseline | Treatment | Change from Baseline | P vs. Zero Change |
|---|--------------|--------------|----------------------|-------------------|
| <i>Leg press strength (kg) (overall ANOVA P = 0.0003)</i> | | | | |
| 25 mg | 355.5 ± 31.3 | 354.2 ± 27.9 | -1.2 ± 7.4 | 0.8701 |
| 50 mg | 407.8 ± 22.0 | 430.5 ± 22.3 | +22.7 ± 7.6 | 0.0204 |
| 125 mg | 419.2 ± 24.4 | 444.6 ± 32.2 | +18.4 ± 10.0 | 0.4195 |
| 300 mg | 439.8 ± 25.7 | 525.5 ± 24.9 | +72.2 ± 12.4 | 0.0004 |
| 600 mg | 431.6 ± 27.6 | 508.1 ± 28.1 | +76.5 ± 12.2 | 0.0001 |
| <i>Leg power (W) (overall ANOVA P = 0.0419)</i> | | | | |
| 25 mg | 183.6 ± 10.6 | 188.9 ± 12.9 | 5.3 ± 8.4 | 0.5429 |
| 50 mg | 234.4 ± 14.2 | 249.6 ± 17.8 | 15.2 ± 15.0 | 0.3468 |
| 125 mg | 253.8 ± 20.6 | 265.6 ± 25.2 | 8.5 ± 15.3 | 0.5935 |
| 300 mg | 233.8 ± 20.2 | 272.4 ± 27.8 | 38.6 ± 9.4 | 0.0033 |
| 600 mg | 212.4 ± 11.0 | 256.2 ± 13.8 | 48.1 ± 11.8 | 0.0015 |

Values on each day represent the mean (±SE) of all available values on that day. However, the change represents the difference between paired values only.

Table 6. *Change in scores for sexual activity, sexual desire, and spatial cognition*

| Testosterone Dose | Baseline | Treatment | Change from Baseline | P vs. zero change |
|--|------------|------------|----------------------|-------------------|
| <i>Sexual activity scores (overall ANOVA P = 0.7842)</i> | | | | |
| 25 mg | 10.7 ± 1.7 | 8.2 ± 2.9 | -2.5 ± 3.2 | 0.4729 |
| 50 mg | 14.1 ± 2.1 | 13.7 ± 1.8 | -0.4 ± 2.8 | 0.9017 |
| 125 mg | 9.8 ± 2.7 | 12.0 ± 2.9 | 2.2 ± 3.1 | 0.5151 |
| 300 mg | 11.6 ± 1.6 | 12.0 ± 1.9 | 0.7 ± 0.9 | 0.4761 |
| 600 mg | 16.1 ± 3.7 | 15.6 ± 0.5 | 0.7 ± 2.2 | 0.7891 |
| <i>Intensity of sexual desire scores (overall ANOVA P = 0.477)</i> | | | | |
| 25 mg | 1.9 ± 0.1 | 1.3 ± 0.4 | -0.6 ± 0.4 | 0.2253 |
| 50 mg | 2.3 ± 0.1 | 2.2 ± 0.3 | -0.0 ± 0.3 | 0.9615 |
| 125 mg | 2.1 ± 0.1 | 2.0 ± 0.3 | -0.1 ± 0.4 | 0.9078 |
| 300 mg | 2.2 ± 0.2 | 2.4 ± 0.2 | 0.1 ± 0.1 | 0.3559 |
| 600 mg | 2.7 ± 0.2 | 2.2 ± 0.1 | 0.2 ± 0.2 | 0.4075 |
| <i>Spatial cognition scores</i> | | | | |
| <i>1. No. of trial levels on the checkerboard test that the participant reached before the test was terminated (overall ANOVA P = 0.235)</i> | | | | |
| 25 mg | 6.8 ± 0.3 | 6.4 ± 0.3 | -0.4 ± 0.3 | 0.284 |
| 50 mg | 6.7 ± 0.3 | 6.7 ± 0.3 | 0.3 ± 0.3 | 0.284 |
| 125 mg | 6.6 ± 0.3 | 6.6 ± 0.2 | 0.0 ± 0.4 | 1.0 |
| 300 mg | 7.3 ± 0.2 | 6.7 ± 0.2 | -0.6 ± 0.3 | 0.103 |
| 600 mg | 6.6 ± 0.2 | 6.9 ± 0.2 | 0.3 ± 0.3 | 0.278 |
| <i>2. No. of checkerboard squares correctly marked in all trials (overall ANOVA P = 0.6309)</i> | | | | |
| 25 mg | 28.6 ± 2.2 | 30.4 ± 2.1 | 1.8 ± 2.1 | 0.4272 |
| 50 mg | 30.0 ± 2.3 | 34.7 ± 4.9 | 2.7 ± 3.5 | 0.5236 |
| 125 mg | 27.3 ± 3.0 | 28.1 ± 2.2 | 0.9 ± 3.8 | 0.7292 |
| 300 mg | 32.6 ± 2.1 | 33.3 ± 1.8 | 0.7 ± 3.1 | 0.8241 |
| 600 mg | 26.7 ± 2.7 | 32.5 ± 2.1 | 5.8 ± 2.2 | 0.0265 |

Values are means ± SE.

Serum testosterone levels were measured 7 days after previous injection; they reflect the lowest testosterone levels after an injection. Testosterone concentrations were higher at other time points. Weekly injections of testosterone enanthate are associated with fluctuations in testosterone levels (44). Although nadir testosterone concentrations were highly correlated with testosterone enanthate dose, it is possible that sustained testosterone delivery by a patch or gel might reveal different dose-response relationships, particularly with respect to hemoglobin and HDL cholesterol (19).

There were no significant changes in overall sexual activity or sexual desire in any group, including those receiving the 25-mg dose. Testosterone replacement of hypogonadal men improves frequency of sexual acts and fantasies, sexual desire, and response to visual erotic stimuli (3, 13, 15, 17, 31, 41). Our data demonstrate that serum testosterone concentrations at the lower end of male range can maintain some aspects of sexual function (3, 13). Testosterone has been shown to regulate nitric oxide synthase activity in the cavernosal smooth muscle (32), and it is possible that optimum penile rigidity might require higher testosterone levels than those produced by the 25-mg dose.

This study demonstrates that an increase in circulating testosterone concentrations results in dose-de-

Table 7. Changes in hemoglobin, plasma HDL cholesterol, and PSA

| Testosterone Dose | Baseline | Week 20 | Change from Baseline | P vs. Zero Change |
|--|-------------|-------------|----------------------|-------------------|
| <i>Hemoglobin (g/l), (overall ANOVA P = 0.0001)</i> | | | | |
| 25 mg | 143.5 ± 3.5 | 139.0 ± 2.5 | -5.2 ± 3.5 | 0.1759 |
| 50 mg | 150.8 ± 3.3 | 146.6 ± 2.0 | -7.4 ± 2.3 | 0.0153 |
| 125 mg | 141.9 ± 2.6 | 146.1 ± 3.1 | 2.5 ± 2.4 | 0.3061 |
| 300 mg | 143.5 ± 2.2 | 149.6 ± 3.1 | 6.1 ± 2.9 | 0.0639 |
| 600 mg | 141.5 ± 2.3 | 155.7 ± 2.2 | 14.2 ± 2.0 | 0.0001 |
| <i>PSA (ng/ml), (overall ANOVA P = 0.5290)</i> | | | | |
| 25 mg | 1.0 ± 0.2 | 1.0 ± 0.2 | -0.1 ± 0.2 | 0.6870 |
| 50 mg | 0.8 ± 0.1 | 1.1 ± 0.2 | 0.3 ± 0.1 | 0.0186 |
| 125 mg | 0.7 ± 0.1 | 0.8 ± 0.1 | 0.1 ± 0.1 | 0.1721 |
| 300 mg | 0.7 ± 0.1 | 0.9 ± 0.3 | 0.2 ± 0.2 | 0.4525 |
| 600 mg | 0.5 ± 0.1 | 0.7 ± 0.1 | 0.1 ± 0.0 | 0.0010 |
| <i>Plasma HDL cholesterol (mg/dl) (overall ANOVA P = 0.0049)</i> | | | | |
| 25 mg | 46 ± 3 | 51 ± 4 | +4.5 ± 2.6 | 0.1202 |
| 50 mg | 48 ± 3 | 47 ± 5 | -0.7 ± 4.0 | 0.8653 |
| 125 mg | 48 ± 2 | 43 ± 3 | -4.0 ± 1.7 | 0.0476 |
| 300 mg | 47 ± 3 | 41 ± 2 | -5.7 ± 2.8 | 0.0690 |
| 600 mg | 43 ± 2 | 34 ± 2 | -8.4 ± 1.8 | 0.0005 |

Values on each day represent the mean (\pm SE) of all available values on that day. However, the change from baseline represents the difference between paired values only. PSA, prostate-specific antigen; HDL, high-density lipoprotein.

pendent increases in fat-free mass, muscle size, strength, and power. The relationships between circulating testosterone concentrations and changes in fat-free mass and muscle size conform to a single log-linear dose-response curve. Our data do not support the notion of two separate dose-response curves reflecting two independent mechanisms of testosterone action on the muscle. Forbes et al. (22) predicted 25 years ago that the muscle mass accretion during androgen administration is related to the cumulative androgen dose, the product of daily dose and treatment duration. Our data are consistent with Forbes's hypothesis of a linear relationship between testosterone dose and lean mass accretion; however, we do not know whether increasing the treatment duration would lead to further gains in muscle mass.

In addition, we do not know whether responsiveness to testosterone is attenuated in older men. Testosterone dose-response relationships might be modulated by other muscle growth regulators, such as nutritional status, exercise and activity level, glucocorticoids, thyroid hormones, and endogenous growth hormone secretory status.

Serum PSA levels decrease after androgen withdrawal, and testosterone replacement of hypogonadal men increases PSA levels into the normal range (16, 34). We did not find significant changes in PSA at any dose, indicating that the lowest dose of testosterone maintained PSA levels. We did not measure prostate volume in this study; therefore, we do not know whether prostate volume exhibits the same relationship with testosterone dose as PSA levels.

Hemoglobin levels changed significantly in relation to testosterone dose and concentration. Testosterone

regulates erythropoiesis through its effects on erythropoietin and stem cell proliferation (14, 35, 40). Although modest increments in hemoglobin might be beneficial in androgen-deficient men with chronic illness who are anemic, marked increases in hemoglobin levels could increase the risk of cerebrovascular events (25) and hypertension (42).

Although men, on average, perform better on tests of spatial cognition than women, testosterone replacement has not been consistently shown to improve spatial cognition in hypogonadal men (1, 29, 48). We did not find changes in spatial cognition at any dose. The effect size of gender differences in spatial cognition is small; it is possible that our study did not have sufficient power to detect small differences. We cannot exclude the possibility that gender differences in spatial cognition might be due to organizational effects of testosterone and might not respond to changes in testosterone levels in adult men.

Although mean change in fat-free mass and muscle size correlated with testosterone dose and concentration, there was considerable heterogeneity in response to testosterone administration within each group. These individual differences in response to androgen administration might reflect differences in activity level, testosterone metabolism, nutrition, or polymorphisms in androgen receptor, myostatin, 5- α -reductase, or other muscle growth regulators.

Our data demonstrate that different androgen-dependent processes have different testosterone dose-response relationships. Some aspects of sexual function and spatial cognition, and PSA levels, were maintained by relatively low doses of testosterone in GnRH agonist-treated men and did not increase further with administration of higher doses of testosterone. In contrast, graded doses of testosterone were associated with dose and testosterone concentration-dependent changes in fat-free mass, fat mass, muscle volume, leg press strength and power, hemoglobin, IGF-I, and plasma HDL cholesterol. The precise mechanisms for the tissue- and function-specific differences in testosterone dose dependence are not well understood (36). Although only a single androgen receptor protein is expressed in all androgen-responsive tissues, tissue specificity of androgen action might be mediated through combinatorial recruitment of tissue-specific coactivators and corepressors (36).

Testosterone doses associated with significant gains in fat-free mass, muscle size, and strength were associated with significant reductions in plasma HDL concentrations. Further studies are needed to determine whether clinically significant anabolic effects of testosterone can be achieved without adversely affecting cardiovascular risk. Selective androgen receptor modulators that preferentially augment muscle mass and strength, but only minimally affect prostate and cardiovascular risk factors, are desirable (36).

This study was supported primarily by National Institutes of Health (NIH) Grant 1R01-AG-14369; additional support was pro-

vided by Grants 1R01-DK-49296, 1R01-DK-59297-01, Federal Drug Administration Grant ODP 1397, a General Clinical Research Center Grant MO-00425, NIH-National Center for Research Resources-00954, RCMi Grants P20-RR-11145-01 (RCMi Clinical Research Initiative) and G12-RR-03026. BioTechnology General (Iselin, NJ) provided testosterone enanthate, and R. P. Debio (Martigny, Switzerland) provided the GnRH agonist (Decapeptyl).

REFERENCES

- Alexander GM, Swerdloff RS, Wang C, Davidson T, McDonald V, Steiner B, and Hines M. Androgen-behavior correlations in hypogonadal men and eugonadal men. II. Cognitive abilities. *Horm Behav* 33: 85–94, 1998.
- Antonio J, Wilson JD, and George FW. Effects of castration and androgen treatment on androgen-receptor levels in rat skeletal muscles. *J Appl Physiol* 87: 2016–2019, 1999.
- Bagatell CJ, Heiman JR, Rivier JE, and Bremner WJ. Effects of endogenous testosterone and estradiol on sexual behavior in normal young men. *J Clin Endocrinol Metab* 78: 711–716, 1994.
- Bassey EJ, Fiatarone MA, O'Neill EF, Kelly M, Evans WJ, and Lipsitz LA. Leg extensor power and functional performance in very old men and women. *Clin Sci (Colch)* 82: 321–327, 1992.
- Bassey EJ and Short AH. A new method for measuring power output in a single leg extension: feasibility, reliability and validity. *Eur J Appl Physiol* 60: 385–390, 1990.
- Bhasin S. The dose-dependent effects of testosterone on sexual function and on muscle mass and function. *Mayo Clin Proc* 75, Suppl: S70–S75, 2000.
- Bhasin S and Bremner WJ. Clinical review 85: emerging issues in androgen replacement therapy. *J Clin Endocrinol Metab* 82: 3–8, 1997.
- Bhasin S, Fielder TJ, Peacock N, Sod-Moriah UA, and Swerdloff RS. Dissociating antifertility effects of GnRH-antagonist from its adverse effects on mating behavior in male rats. *Am J Physiol Endocrinol Metab* 254: E84–E91, 1988.
- Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Tricker R, Shirazi A, and Casaburi R. The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med* 335: 1–6, 1996.
- Bhasin S, Storer TW, Berman N, Yarasheski KE, Clevenger B, and Casaburi RA. A replacement dose of testosterone increases fat-free mass and muscle size in hypogonadal men. *J Clin Endocrinol Metab* 82: 407–413, 1997.
- Bhasin S, Storer TW, Javanbakht M, Berman N, Yarasheski KE, Phillips J, Dike M, Sinha-Hikim I, Shen R, Hays RD, and Beall G. Testosterone replacement and resistance exercise in HIV-infected men with weight loss and low testosterone levels. *JAMA* 283: 763–770, 2000.
- Brodsky IG, Balagopal P, and Nair KS. Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men—a clinical research center study. *J Clin Endocrinol Metab* 81: 3469–3475, 1996.
- Buena F, Swerdloff RS, Steiner BS, Lutchmansingh P, Peterson MA, Pandian MR, Galmarini M, and Bhasin S. Sexual function does not change when serum testosterone levels are pharmacologically varied within the normal male range. *Fertil Steril* 59: 1118–1123, 1993.
- Byron JW. Effect of steroids on the cycling of haemopoietic stem cells. *Nature* 228: 1204–1206, 1970.
- Carani C, Granata AR, Bancroft J, and Marrama P. The effects of testosterone replacement on nocturnal penile tumescence and rigidity and erectile response to visual erotic stimuli in hypogonadal men. *Psychoneuroendocrinology* 20: 743–753, 1995.
- Cooper CS, Perry PJ, Sparks AE, MacIndoe JH, Yates WR, and Williams RD. Effect of exogenous testosterone on prostate volume, serum and semen prostate specific antigen levels in healthy young men. *J Urol* 159: 441–443, 1998.
- Cunningham GR, Hirshkowitz M, Korenman SG, and Karacan I. Testosterone replacement therapy and sleep-related erections in hypogonadal men. *J Clin Endocrinol Metab* 70: 792–797, 1990.
- Dahlberg E, Snochowski M, and Gustafsson J-A. Regulation of androgen and glucocorticoid receptors in rat and mouse skeletal muscle cytosol. *Endocrinology* 108: 1431–1440, 1981.
- Dobs AS, Meikle AW, Arver S, Sanders SW, Caramelli KE, and Mazer NA. Pharmacokinetics, efficacy, and safety of a permeation-enhanced testosterone transdermal system compared with bi-weekly injections of testosterone enanthate for the treatment of hypogonadal men. *J Clin Endocrinol Metab* 84: 3469–3478, 1999.
- Faries D, Herrera J, Rayamajhi J, DeBrotta D, Demitrack M, and Potter WZ. The responsiveness of the Hamilton Depression Rating Scale. *J Psychiatr Res* 34: 3–10, 2000.
- Fielder TJ, Peacock NR, McGivern RF, Swerdloff RS, and Bhasin S. Testosterone dose-dependency of sexual and nonsexual behaviors in the gonadotropin-releasing hormone antagonist-treated male rat. *J Androl* 10: 167–173, 1989.
- Forbes GB. The effect of anabolic steroids on lean body mass: the dose response curve. *Metabolism* 34: 571–573, 1985.
- Forbes GB, Porta CR, Herr BE, and Griggs RC. Sequence of changes in body composition induced by testosterone and reversal of changes after drug is stopped. *JAMA* 267: 397–399, 1992.
- Fristad MA, Weller RA, and Weller EB. The Mania Rating Scale (MRS): further reliability and validity studies with children. *Ann Clin Psychiatry* 7: 127–132, 1995.
- Gillum RF and Sempos CT. Hemoglobin, hematocrit, and stroke incidence and mortality in women and men. *Stroke* 27: 1910–1914, 1996.
- Griggs RC, Kingston W, Jozefowicz RF, Herr BE, Forbes G, and Halliday D. Effect of testosterone on muscle mass and muscle protein synthesis. *J Appl Physiol* 66: 498–503, 1989.
- Grinspoon S, Corcoran C, Askari H, Schoenfeld D, Wolf L, Burrows B, Walsh M, Hayden D, Parلمان K, Anderson E, Basgoz N, and Klibanski A. Effects of androgen administration in men with the AIDS wasting syndrome: a randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 129: 18–26, 1998.
- Hornum M, Cooper DM, Brasel JA, Bueno A, and Sietsema KE. Exercise-induced changes in circulating growth factors with cyclic variation in plasma estradiol in women. *J Appl Physiol* 82: 1946–1951, 1997.
- Janowsky JS, Oviatt SK, and Orwoll ES. Testosterone influences spatial cognition in older men. *Behav Neurosci* 108: 325–332, 1994.
- Katznelson L, Finkelstein JS, Schoenfeld DA, Rosenthal DI, Anderson EJ, and Klibanski A. Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism. *J Clin Endocrinol Metab* 81: 4358–4365, 1996.
- Kwan M, Greenleaf WJ, Mann J, Crapo L, and Davidson JM. The nature of androgen action on male sexuality: a combined laboratory- self-report study on hypogonadal men. *J Clin Endocrinol Metab* 57: 557–562, 1983.
- Lugg JA, Rajfer J, and Gonzalez-Cadavid NF. Dihydrotestosterone is the active androgen in the maintenance of nitric oxide-mediated penile erection in the rat. *Endocrinology* 136: 1495–1501, 1995.
- Mauras N, Hayes V, Welch S, Veldhuis J, and Urban R. Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. *J Clin Endocrinol Metab* 83: 1886–1892, 1998.
- Meikle AW, Arver S, Dobs AS, Adolfsson J, Sanders SW, Middleton RG, Stephenson RA, Hoover DR, Rajaram L, and Mazer NA. Prostate size in hypogonadal men treated with a nonscrotal permeation-enhanced testosterone transdermal system. *Urology* 49: 191–196, 1997.
- Naets JP and Wittek M. Mechanism of action of androgens on erythropoiesis. *Am J Physiol* 210: 315–320, 1966.
- Negro-Vilar A. Selective androgen receptor modulators (SARMs): a novel approach to androgen therapy for the new millennium. *J Clin Endocrinol Metab* 84: 3459–3462, 1999.
- Pavlou SN, Brewer K, Farley MG, Lindner J, Bastias MC, Rogers BJ, Swift LL, Rivier JE, Vale WW, and Conn PM. Combined administration of a gonadotropin-releasing hormone antagonist and testosterone in men induces reversible azoospermia.

- mia without loss of libido. *J Clin Endocrinol Metab* 73: 1360–1369, 1991.
38. Pope HG and Jacobs A. Evidence for sex-specific residual effect of cannabis on visuo-spatial memory. *Psychother Psychosom* 66: 179–184, 1997.
 39. Rance NE and Max SR. Modulation of the cytosolic androgen receptor in striated muscle by sex-steroids. *Endocrinology* 115: 862–866, 1984.
 40. Rencricca NJ, Solomon J, Fimian WJ Jr, Howard D, Rizzoli V, and Stohman F Jr. The effect of testosterone on erythropoiesis. *Scand J Haematol* 6: 431–436, 1969.
 41. Salmimies P, Kockott G, Pirke KM, Vogt HJ, and Schill WB. Effects of testosterone replacement on sexual behavior in hypogonadal men. *Arch Sex Behav* 11: 345–353, 1982.
 42. Siebers RW, Carter JM, and Maling TJ. Increase in haematocrit in borderline hypertensive men. *Clin Exp Pharmacol Physiol* 21: 401–403, 1994.
 43. Sinha-Hikim I, Arver S, Beall G, Shen R, Guerrero M, Sattler F, Shikuma C, Nelson JC, Landgren BM, Mazer NA, and Bhasin S. The use of a sensitive, equilibrium dialysis method for the measurement of free testosterone levels in healthy, cycling women, and in HIV-infected women. *J Clin Endocrinol Metab* 83: 1312–1318, 1998.
 44. Snyder PJ and Lawrence DA. Treatment of male hypogonadism with testosterone enanthate. *J Clin Endocrinol Metab* 51: 1335–1339, 1980.
 45. Snyder PJ, Peachey H, Berlin JA, Hannoush P, Haddad G, Dlewati A, Santanna J, Loh L, Lenrow DA, Holmes JH, Kapoor SC, Atkinson JE, and Strom BLE. Effects of testosterone replacement in hypogonadal men. *J Clin Endocrinol Metab* 85: 2670–2677, 2000.
 46. Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Lenrow DA, Holmes JH, Dlewati A, Santanna J, Rosen CJ, and Strom BL. Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. *J Clin Endocrinol Metab* 84: 2647–2653, 1999.
 47. Tenover JS. Androgen replacement therapy to reverse and/or prevent age-associated sarcopenia in men. *Bailliere's Clin Endocrinol Metab* 12: 419–425, 1998.
 48. Van Goozen SH, Cohen-Kettenis PT, Gooren LJ, Frijda NH, and Van de Poll NE. Activating effects of androgens on cognitive performance: causal evidence in a group of female-to-male transsexuals. *Neuropsychologia* 32: 1153–1157, 1994.
 49. Wang C, Swedloff RS, Iranmanesh A, Dobs A, Snyder PJ, Cunningham G, Matsumoto AM, Weber T, and Berman N. Transdermal testosterone gel improves sexual function, mood, muscle strength, and body composition parameters in hypogonadal men. Testosterone Gel Study Group. *J Clin Endocrinol Metab* 85: 2839–2853, 2000.
 50. Wilson JD. Androgen abuse by athletes. *Endocr Rev* 9: 181–199, 1988.



General requirements for the competence of testing and calibration laboratories

*Exigences générales concernant la compétence des laboratoires
d'étalonnages et d'essais*

PDF disclaimer

This PDF file may contain embedded typefaces. In accordance with Adobe's licensing policy, this file may be printed or viewed but shall not be edited unless the typefaces which are embedded are licensed to and installed on the computer performing the editing. In downloading this file, parties accept therein the responsibility of not infringing Adobe's licensing policy. The ISO Central Secretariat accepts no liability in this area.

Adobe is a trademark of Adobe Systems Incorporated.

Details of the software products used to create this PDF file can be found in the General Info relative to the file; the PDF-creation parameters were optimized for printing. Every care has been taken to ensure that the file is suitable for use by ISO member bodies. In the unlikely event that a problem relating to it is found, please inform the Central Secretariat at the address given below.

© ISO 2005

All rights reserved. Unless otherwise specified, no part of this publication may be reproduced or utilized in any form or by any means, electronic or mechanical, including photocopying and microfilm, without permission in writing from either ISO at the address below or ISO's member body in the country of the requester.

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

| | |
|---|----|
| Foreword..... | v |
| Introduction | vi |
| 1 Scope..... | 1 |
| 2 Normative references | 2 |
| 3 Terms and definitions..... | 2 |
| 4 Management requirements | 2 |
| 4.1 Organization | 2 |
| 4.2 Management system..... | 3 |
| 4.3 Document control | 4 |
| 4.3.1 General | 4 |
| 4.3.2 Document approval and issue | 4 |
| 4.3.3 Document changes | 5 |
| 4.4 Review of requests, tenders and contracts | 5 |
| 4.5 Subcontracting of tests and calibrations | 6 |
| 4.6 Purchasing services and supplies | 6 |
| 4.7 Service to the customer | 6 |
| 4.8 Complaints..... | 7 |
| 4.9 Control of nonconforming testing and/or calibration work..... | 7 |
| 4.10 Improvement..... | 7 |
| 4.11 Corrective action..... | 8 |
| 4.11.1 General | 8 |
| 4.11.2 Cause analysis | 8 |
| 4.11.3 Selection and implementation of corrective actions..... | 8 |
| 4.11.4 Monitoring of corrective actions | 8 |
| 4.11.5 Additional audits | 8 |
| 4.12 Preventive action | 8 |
| 4.13 Control of records | 9 |
| 4.13.1 General | 9 |
| 4.13.2 Technical records | 9 |
| 4.14 Internal audits..... | 9 |
| 4.15 Management reviews | 10 |
| 5 Technical requirements..... | 10 |
| 5.1 General..... | 10 |
| 5.2 Personnel..... | 11 |
| 5.3 Accommodation and environmental conditions | 12 |
| 5.4 Test and calibration methods and method validation..... | 12 |
| 5.4.1 General | 12 |
| 5.4.2 Selection of methods..... | 13 |
| 5.4.3 Laboratory-developed methods | 13 |
| 5.4.4 Non-standard methods..... | 13 |
| 5.4.5 Validation of methods..... | 14 |
| 5.4.6 Estimation of uncertainty of measurement..... | 14 |
| 5.4.7 Control of data..... | 15 |
| 5.5 Equipment..... | 15 |
| 5.6 Measurement traceability | 17 |
| 5.6.1 General | 17 |
| 5.6.2 Specific requirements..... | 17 |
| 5.6.3 Reference standards and reference materials | 18 |
| 5.7 Sampling | 19 |

5.8 Handling of test and calibration items19

5.9 Assuring the quality of test and calibration results20

5.10 Reporting the results20

5.10.1 General20

5.10.2 Test reports and calibration certificates20

5.10.3 Test reports21

5.10.4 Calibration certificates22

5.10.5 Opinions and interpretations22

5.10.6 Testing and calibration results obtained from subcontractors23

5.10.7 Electronic transmission of results23

5.10.8 Format of reports and certificates23

5.10.9 Amendments to test reports and calibration certificates23

Annex A (informative) Nominal cross-references to ISO 9001:200024

Annex B (informative) Guidelines for establishing applications for specific fields26

Bibliography27

Foreword

ISO (the International Organization for Standardization) and IEC (the International Electrotechnical Commission) form the specialized system for worldwide standardization. National bodies that are members of ISO or IEC participate in the development of International Standards through technical committees established by the respective organization to deal with particular fields of technical activity. ISO and IEC technical committees collaborate in fields of mutual interest. Other international organizations, governmental and non-governmental, in liaison with ISO and IEC, also take part in the work. In the field of conformity assessment, the ISO Committee on conformity assessment (CASCO) is responsible for the development of International Standards and Guides.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

Draft International Standards are circulated to the national bodies for voting. Publication as an International Standard requires approval by at least 75 % of the national bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/IEC 17025 was prepared by the *ISO Committee on conformity assessment (CASCO)*.

It was circulated for voting to the national bodies of both ISO and IEC, and was approved by both organizations.

This second edition cancels and replaces the first edition (ISO/IEC 17025:1999), which has been technically revised.

Introduction

The first edition (1999) of this International Standard was produced as the result of extensive experience in the implementation of ISO/IEC Guide 25 and EN 45001, both of which it replaced. It contained all of the requirements that testing and calibration laboratories have to meet if they wish to demonstrate that they operate a management system, are technically competent, and are able to generate technically valid results.

The first edition referred to ISO 9001:1994 and ISO 9002:1994. These standards have been superseded by ISO 9001:2000, which made an alignment of ISO/IEC 17025 necessary. In this second edition, clauses have been amended or added only when considered necessary in the light of ISO 9001:2000.

Accreditation bodies that recognize the competence of testing and calibration laboratories should use this International Standard as the basis for their accreditation. Clause 4 specifies the requirements for sound management. Clause 5 specifies the requirements for technical competence for the type of tests and/or calibrations the laboratory undertakes.

Growth in the use of management systems generally has increased the need to ensure that laboratories which form part of larger organizations or offer other services can operate to a quality management system that is seen as compliant with ISO 9001 as well as with this International Standard. Care has been taken, therefore, to incorporate all those requirements of ISO 9001 that are relevant to the scope of testing and calibration services that are covered by the laboratory's management system.

Testing and calibration laboratories that comply with this International Standard will therefore also operate in accordance with ISO 9001.

Conformity of the quality management system within which the laboratory operates to the requirements of ISO 9001 does not of itself demonstrate the competence of the laboratory to produce technically valid data and results. Nor does demonstrated conformity to this International Standard imply conformity of the quality management system within which the laboratory operates to all the requirements of ISO 9001.

The acceptance of testing and calibration results between countries should be facilitated if laboratories comply with this International Standard and if they obtain accreditation from bodies which have entered into mutual recognition agreements with equivalent bodies in other countries using this International Standard.

The use of this International Standard will facilitate cooperation between laboratories and other bodies, and assist in the exchange of information and experience, and in the harmonization of standards and procedures.

General requirements for the competence of testing and calibration laboratories

1 Scope

1.1 This International Standard specifies the general requirements for the competence to carry out tests and/or calibrations, including sampling. It covers testing and calibration performed using standard methods, non-standard methods, and laboratory-developed methods.

1.2 This International Standard is applicable to all organizations performing tests and/or calibrations. These include, for example, first-, second- and third-party laboratories, and laboratories where testing and/or calibration forms part of inspection and product certification.

This International Standard is applicable to all laboratories regardless of the number of personnel or the extent of the scope of testing and/or calibration activities. When a laboratory does not undertake one or more of the activities covered by this International Standard, such as sampling and the design/development of new methods, the requirements of those clauses do not apply.

1.3 The notes given provide clarification of the text, examples and guidance. They do not contain requirements and do not form an integral part of this International Standard.

1.4 This International Standard is for use by laboratories in developing their management system for quality, administrative and technical operations. Laboratory customers, regulatory authorities and accreditation bodies may also use it in confirming or recognizing the competence of laboratories. This International Standard is not intended to be used as the basis for certification of laboratories.

NOTE 1 The term 'management system' in this International Standard means the quality, administrative and technical systems that govern the operations of a laboratory.

NOTE 2 Certification of a management system is sometimes also called registration.

1.5 Compliance with regulatory and safety requirements on the operation of laboratories is not covered by this International Standard.

1.6 If testing and calibration laboratories comply with the requirements of this International Standard, they will operate a quality management system for their testing and calibration activities that also meets the principles of ISO 9001. Annex A provides nominal cross-references between this International Standard and ISO 9001. This International Standard covers technical competence requirements that are not covered by ISO 9001.

NOTE 1 It might be necessary to explain or interpret certain requirements in this International Standard to ensure that the requirements are applied in a consistent manner. Guidance for establishing applications for specific fields, especially for accreditation bodies (see ISO/IEC 17011) is given in Annex B.

NOTE 2 If a laboratory wishes accreditation for part or all of its testing and calibration activities, it should select an accreditation body that operates in accordance with ISO/IEC 17011.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/IEC 17000, *Conformity assessment — Vocabulary and general principles*

VIM, *International vocabulary of basic and general terms in metrology*, issued by BIPM, IEC, IFCC, ISO, IUPAC, IUPAP and OIML

NOTE Further related standards, guides, etc. on subjects included in this International Standard are given in the Bibliography.

3 Terms and definitions

For the purposes of this document, the relevant terms and definitions given in ISO/IEC 17000 and VIM apply.

NOTE General definitions related to quality are given in ISO 9000, whereas ISO/IEC 17000 gives definitions specifically related to certification and laboratory accreditation. Where different definitions are given in ISO 9000, the definitions in ISO/IEC 17000 and VIM are preferred.

4 Management requirements

4.1 Organization

4.1.1 The laboratory or the organization of which it is part shall be an entity that can be held legally responsible.

4.1.2 It is the responsibility of the laboratory to carry out its testing and calibration activities in such a way as to meet the requirements of this International Standard and to satisfy the needs of the customer, the regulatory authorities or organizations providing recognition.

4.1.3 The management system shall cover work carried out in the laboratory's permanent facilities, at sites away from its permanent facilities, or in associated temporary or mobile facilities.

4.1.4 If the laboratory is part of an organization performing activities other than testing and/or calibration, the responsibilities of key personnel in the organization that have an involvement or influence on the testing and/or calibration activities of the laboratory shall be defined in order to identify potential conflicts of interest.

NOTE 1 Where a laboratory is part of a larger organization, the organizational arrangements should be such that departments having conflicting interests, such as production, commercial marketing or financing do not adversely influence the laboratory's compliance with the requirements of this International Standard.

NOTE 2 If the laboratory wishes to be recognized as a third-party laboratory, it should be able to demonstrate that it is impartial and that it and its personnel are free from any undue commercial, financial and other pressures which might influence their technical judgement. The third-party testing or calibration laboratory should not engage in any activities that may endanger the trust in its independence of judgement and integrity in relation to its testing or calibration activities.

4.1.5 The laboratory shall

- a) have managerial and technical personnel who, irrespective of other responsibilities, have the authority and resources needed to carry out their duties, including the implementation, maintenance and improvement of the management system, and to identify the occurrence of departures from the management system or from the procedures for performing tests and/or calibrations, and to initiate actions to prevent or minimize such departures (see also 5.2);

- b) have arrangements to ensure that its management and personnel are free from any undue internal and external commercial, financial and other pressures and influences that may adversely affect the quality of their work;
- c) have policies and procedures to ensure the protection of its customers' confidential information and proprietary rights, including procedures for protecting the electronic storage and transmission of results;
- d) have policies and procedures to avoid involvement in any activities that would diminish confidence in its competence, impartiality, judgement or operational integrity;
- e) define the organization and management structure of the laboratory, its place in any parent organization, and the relationships between quality management, technical operations and support services;
- f) specify the responsibility, authority and interrelationships of all personnel who manage, perform or verify work affecting the quality of the tests and/or calibrations;
- g) provide adequate supervision of testing and calibration staff, including trainees, by persons familiar with methods and procedures, purpose of each test and/or calibration, and with the assessment of the test or calibration results;
- h) have technical management which has overall responsibility for the technical operations and the provision of the resources needed to ensure the required quality of laboratory operations;
- i) appoint a member of staff as quality manager (however named) who, irrespective of other duties and responsibilities, shall have defined responsibility and authority for ensuring that the management system related to quality is implemented and followed at all times; the quality manager shall have direct access to the highest level of management at which decisions are made on laboratory policy or resources;
- j) appoint deputies for key managerial personnel (see Note);
- k) ensure that its personnel are aware of the relevance and importance of their activities and how they contribute to the achievement of the objectives of the management system.

NOTE Individuals may have more than one function and it may be impractical to appoint deputies for every function.

4.1.6 Top management shall ensure that appropriate communication processes are established within the laboratory and that communication takes place regarding the effectiveness of the management system.

4.2 Management system

4.2.1 The laboratory shall establish, implement and maintain a management system appropriate to the scope of its activities. The laboratory shall document its policies, systems, programmes, procedures and instructions to the extent necessary to assure the quality of the test and/or calibration results. The system's documentation shall be communicated to, understood by, available to, and implemented by the appropriate personnel.

4.2.2 The laboratory's management system policies related to quality, including a quality policy statement, shall be defined in a quality manual (however named). The overall objectives shall be established, and shall be reviewed during management review. The quality policy statement shall be issued under the authority of top management. It shall include at least the following:

- a) the laboratory management's commitment to good professional practice and to the quality of its testing and calibration in servicing its customers;
- b) the management's statement of the laboratory's standard of service;
- c) the purpose of the management system related to quality;

- d) a requirement that all personnel concerned with testing and calibration activities within the laboratory familiarize themselves with the quality documentation and implement the policies and procedures in their work; and
- e) the laboratory management's commitment to comply with this International Standard and to continually improve the effectiveness of the management system.

NOTE The quality policy statement should be concise and may include the requirement that tests and/or calibrations shall always be carried out in accordance with stated methods and customers' requirements. When the test and/or calibration laboratory is part of a larger organization, some quality policy elements may be in other documents.

4.2.3 Top management shall provide evidence of commitment to the development and implementation of the management system and to continually improving its effectiveness.

4.2.4 Top management shall communicate to the organization the importance of meeting customer requirements as well as statutory and regulatory requirements.

4.2.5 The quality manual shall include or make reference to the supporting procedures including technical procedures. It shall outline the structure of the documentation used in the management system.

4.2.6 The roles and responsibilities of technical management and the quality manager, including their responsibility for ensuring compliance with this International Standard, shall be defined in the quality manual.

4.2.7 Top management shall ensure that the integrity of the management system is maintained when changes to the management system are planned and implemented.

4.3 Document control

4.3.1 General

The laboratory shall establish and maintain procedures to control all documents that form part of its management system (internally generated or from external sources), such as regulations, standards, other normative documents, test and/or calibration methods, as well as drawings, software, specifications, instructions and manuals.

NOTE 1 In this context "document" could be policy statements, procedures, specifications, calibration tables, charts, text books, posters, notices, memoranda, software, drawings, plans, etc. These may be on various media, whether hard copy or electronic, and they may be digital, analog, photographic or written.

NOTE 2 The control of data related to testing and calibration is covered in 5.4.7. The control of records is covered in 4.13.

4.3.2 Document approval and issue

4.3.2.1 All documents issued to personnel in the laboratory as part of the management system shall be reviewed and approved for use by authorized personnel prior to issue. A master list or an equivalent document control procedure identifying the current revision status and distribution of documents in the management system shall be established and shall be readily available to preclude the use of invalid and/or obsolete documents.

4.3.2.2 The procedure(s) adopted shall ensure that:

- a) authorized editions of appropriate documents are available at all locations where operations essential to the effective functioning of the laboratory are performed;
- b) documents are periodically reviewed and, where necessary, revised to ensure continuing suitability and compliance with applicable requirements;

- c) invalid or obsolete documents are promptly removed from all points of issue or use, or otherwise assured against unintended use;
- d) obsolete documents retained for either legal or knowledge preservation purposes are suitably marked.

4.3.2.3 Management system documents generated by the laboratory shall be uniquely identified. Such identification shall include the date of issue and/or revision identification, page numbering, the total number of pages or a mark to signify the end of the document, and the issuing authority(ies).

4.3.3 Document changes

4.3.3.1 Changes to documents shall be reviewed and approved by the same function that performed the original review unless specifically designated otherwise. The designated personnel shall have access to pertinent background information upon which to base their review and approval.

4.3.3.2 Where practicable, the altered or new text shall be identified in the document or the appropriate attachments.

4.3.3.3 If the laboratory's document control system allows for the amendment of documents by hand pending the re-issue of the documents, the procedures and authorities for such amendments shall be defined. Amendments shall be clearly marked, initialled and dated. A revised document shall be formally re-issued as soon as practicable.

4.3.3.4 Procedures shall be established to describe how changes in documents maintained in computerized systems are made and controlled.

4.4 Review of requests, tenders and contracts

4.4.1 The laboratory shall establish and maintain procedures for the review of requests, tenders and contracts. The policies and procedures for these reviews leading to a contract for testing and/or calibration shall ensure that:

- a) the requirements, including the methods to be used, are adequately defined, documented and understood (see 5.4.2);
- b) the laboratory has the capability and resources to meet the requirements;
- c) the appropriate test and/or calibration method is selected and is capable of meeting the customers' requirements (see 5.4.2).

Any differences between the request or tender and the contract shall be resolved before any work commences. Each contract shall be acceptable both to the laboratory and the customer.

NOTE 1 The request, tender and contract review should be conducted in a practical and efficient manner, and the effect of financial, legal and time schedule aspects should be taken into account. For internal customers, reviews of requests, tenders and contracts can be performed in a simplified way.

NOTE 2 The review of capability should establish that the laboratory possesses the necessary physical, personnel and information resources, and that the laboratory's personnel have the skills and expertise necessary for the performance of the tests and/or calibrations in question. The review may also encompass results of earlier participation in interlaboratory comparisons or proficiency testing and/or the running of trial test or calibration programmes using samples or items of known value in order to determine uncertainties of measurement, limits of detection, confidence limits, etc.

NOTE 3 A contract may be any written or oral agreement to provide a customer with testing and/or calibration services.

4.4.2 Records of reviews, including any significant changes, shall be maintained. Records shall also be maintained of pertinent discussions with a customer relating to the customer's requirements or the results of the work during the period of execution of the contract.

NOTE For review of routine and other simple tasks, the date and the identification (e.g. the initials) of the person in the laboratory responsible for carrying out the contracted work are considered adequate. For repetitive routine tasks, the review need be made only at the initial enquiry stage or on granting of the contract for on-going routine work performed under a general agreement with the customer, provided that the customer's requirements remain unchanged. For new, complex or advanced testing and/or calibration tasks, a more comprehensive record should be maintained.

4.4.3 The review shall also cover any work that is subcontracted by the laboratory.

4.4.4 The customer shall be informed of any deviation from the contract.

4.4.5 If a contract needs to be amended after work has commenced, the same contract review process shall be repeated and any amendments shall be communicated to all affected personnel.

4.5 Subcontracting of tests and calibrations

4.5.1 When a laboratory subcontracts work, whether because of unforeseen reasons (e.g. workload, need for further expertise or temporary incapacity) or on a continuing basis (e.g. through permanent subcontracting, agency or franchising arrangements), this work shall be placed with a competent subcontractor. A competent subcontractor is one that, for example, complies with this International Standard for the work in question.

4.5.2 The laboratory shall advise the customer of the arrangement in writing and, when appropriate, gain the approval of the customer, preferably in writing.

4.5.3 The laboratory is responsible to the customer for the subcontractor's work, except in the case where the customer or a regulatory authority specifies which subcontractor is to be used.

4.5.4 The laboratory shall maintain a register of all subcontractors that it uses for tests and/or calibrations and a record of the evidence of compliance with this International Standard for the work in question.

4.6 Purchasing services and supplies

4.6.1 The laboratory shall have a policy and procedure(s) for the selection and purchasing of services and supplies it uses that affect the quality of the tests and/or calibrations. Procedures shall exist for the purchase, reception and storage of reagents and laboratory consumable materials relevant for the tests and calibrations.

4.6.2 The laboratory shall ensure that purchased supplies and reagents and consumable materials that affect the quality of tests and/or calibrations are not used until they have been inspected or otherwise verified as complying with standard specifications or requirements defined in the methods for the tests and/or calibrations concerned. These services and supplies used shall comply with specified requirements. Records of actions taken to check compliance shall be maintained.

4.6.3 Purchasing documents for items affecting the quality of laboratory output shall contain data describing the services and supplies ordered. These purchasing documents shall be reviewed and approved for technical content prior to release.

NOTE The description may include type, class, grade, precise identification, specifications, drawings, inspection instructions, other technical data including approval of test results, the quality required and the management system standard under which they were made.

4.6.4 The laboratory shall evaluate suppliers of critical consumables, supplies and services which affect the quality of testing and calibration, and shall maintain records of these evaluations and list those approved.

4.7 Service to the customer

4.7.1 The laboratory shall be willing to cooperate with customers or their representatives in clarifying the customer's request and in monitoring the laboratory's performance in relation to the work performed, provided that the laboratory ensures confidentiality to other customers.

NOTE 1 Such cooperation may include:

- a) providing the customer or the customer's representative reasonable access to relevant areas of the laboratory for the witnessing of tests and/or calibrations performed for the customer;
- b) preparation, packaging, and dispatch of test and/or calibration items needed by the customer for verification purposes.

NOTE 2 Customers value the maintenance of good communication, advice and guidance in technical matters, and opinions and interpretations based on results. Communication with the customer, especially in large assignments, should be maintained throughout the work. The laboratory should inform the customer of any delays or major deviations in the performance of the tests and/or calibrations.

4.7.2 The laboratory shall seek feedback, both positive and negative, from its customers. The feedback shall be used and analysed to improve the management system, testing and calibration activities and customer service.

NOTE Examples of the types of feedback include customer satisfaction surveys and review of test or calibration reports with customers.

4.8 Complaints

The laboratory shall have a policy and procedure for the resolution of complaints received from customers or other parties. Records shall be maintained of all complaints and of the investigations and corrective actions taken by the laboratory (see also 4.11).

4.9 Control of nonconforming testing and/or calibration work

4.9.1 The laboratory shall have a policy and procedures that shall be implemented when any aspect of its testing and/or calibration work, or the results of this work, do not conform to its own procedures or the agreed requirements of the customer. The policy and procedures shall ensure that:

- a) the responsibilities and authorities for the management of nonconforming work are designated and actions (including halting of work and withholding of test reports and calibration certificates, as necessary) are defined and taken when nonconforming work is identified;
- b) an evaluation of the significance of the nonconforming work is made;
- c) correction is taken immediately, together with any decision about the acceptability of the nonconforming work;
- d) where necessary, the customer is notified and work is recalled;
- e) the responsibility for authorizing the resumption of work is defined.

NOTE Identification of nonconforming work or problems with the management system or with testing and/or calibration activities can occur at various places within the management system and technical operations. Examples are customer complaints, quality control, instrument calibration, checking of consumable materials, staff observations or supervision, test report and calibration certificate checking, management reviews and internal or external audits.

4.9.2 Where the evaluation indicates that the nonconforming work could recur or that there is doubt about the compliance of the laboratory's operations with its own policies and procedures, the corrective action procedures given in 4.11 shall be promptly followed.

4.10 Improvement

The laboratory shall continually improve the effectiveness of its management system through the use of the quality policy, quality objectives, audit results, analysis of data, corrective and preventive actions and management review.

4.11 Corrective action

4.11.1 General

The laboratory shall establish a policy and a procedure and shall designate appropriate authorities for implementing corrective action when nonconforming work or departures from the policies and procedures in the management system or technical operations have been identified.

NOTE A problem with the management system or with the technical operations of the laboratory may be identified through a variety of activities, such as control of nonconforming work, internal or external audits, management reviews, feedback from customers and from staff observations.

4.11.2 Cause analysis

The procedure for corrective action shall start with an investigation to determine the root cause(s) of the problem.

NOTE Cause analysis is the key and sometimes the most difficult part in the corrective action procedure. Often the root cause is not obvious and thus a careful analysis of all potential causes of the problem is required. Potential causes could include customer requirements, the samples, sample specifications, methods and procedures, staff skills and training, consumables, or equipment and its calibration.

4.11.3 Selection and implementation of corrective actions

Where corrective action is needed, the laboratory shall identify potential corrective actions. It shall select and implement the action(s) most likely to eliminate the problem and to prevent recurrence.

Corrective actions shall be to a degree appropriate to the magnitude and the risk of the problem.

The laboratory shall document and implement any required changes resulting from corrective action investigations.

4.11.4 Monitoring of corrective actions

The laboratory shall monitor the results to ensure that the corrective actions taken have been effective.

4.11.5 Additional audits

Where the identification of nonconformities or departures casts doubts on the laboratory's compliance with its own policies and procedures, or on its compliance with this International Standard, the laboratory shall ensure that the appropriate areas of activity are audited in accordance with 4.14 as soon as possible.

NOTE Such additional audits often follow the implementation of the corrective actions to confirm their effectiveness. An additional audit should be necessary only when a serious issue or risk to the business is identified.

4.12 Preventive action

4.12.1 Needed improvements and potential sources of nonconformities, either technical or concerning the management system, shall be identified. When improvement opportunities are identified or if preventive action is required, action plans shall be developed, implemented and monitored to reduce the likelihood of the occurrence of such nonconformities and to take advantage of the opportunities for improvement.

4.12.2 Procedures for preventive actions shall include the initiation of such actions and the application of controls to ensure that they are effective.

NOTE 1 Preventive action is a pro-active process to identify opportunities for improvement rather than a reaction to the identification of problems or complaints.

NOTE 2 Apart from the review of the operational procedures, the preventive action might involve analysis of data, including trend and risk analyses and proficiency-testing results.

4.13 Control of records

4.13.1 General

4.13.1.1 The laboratory shall establish and maintain procedures for identification, collection, indexing, access, filing, storage, maintenance and disposal of quality and technical records. Quality records shall include reports from internal audits and management reviews as well as records of corrective and preventive actions.

4.13.1.2 All records shall be legible and shall be stored and retained in such a way that they are readily retrievable in facilities that provide a suitable environment to prevent damage or deterioration and to prevent loss. Retention times of records shall be established.

NOTE Records may be in any media, such as hard copy or electronic media.

4.13.1.3 All records shall be held secure and in confidence.

4.13.1.4 The laboratory shall have procedures to protect and back-up records stored electronically and to prevent unauthorized access to or amendment of these records.

4.13.2 Technical records

4.13.2.1 The laboratory shall retain records of original observations, derived data and sufficient information to establish an audit trail, calibration records, staff records and a copy of each test report or calibration certificate issued, for a defined period. The records for each test or calibration shall contain sufficient information to facilitate, if possible, identification of factors affecting the uncertainty and to enable the test or calibration to be repeated under conditions as close as possible to the original. The records shall include the identity of personnel responsible for the sampling, performance of each test and/or calibration and checking of results.

NOTE 1 In certain fields it may be impossible or impractical to retain records of all original observations.

NOTE 2 Technical records are accumulations of data (see 5.4.7) and information which result from carrying out tests and/or calibrations and which indicate whether specified quality or process parameters are achieved. They may include forms, contracts, work sheets, work books, check sheets, work notes, control graphs, external and internal test reports and calibration certificates, customers' notes, papers and feedback.

4.13.2.2 Observations, data and calculations shall be recorded at the time they are made and shall be identifiable to the specific task.

4.13.2.3 When mistakes occur in records, each mistake shall be crossed out, not erased, made illegible or deleted, and the correct value entered alongside. All such alterations to records shall be signed or initialled by the person making the correction. In the case of records stored electronically, equivalent measures shall be taken to avoid loss or change of original data.

4.14 Internal audits

4.14.1 The laboratory shall periodically, and in accordance with a predetermined schedule and procedure, conduct internal audits of its activities to verify that its operations continue to comply with the requirements of the management system and this International Standard. The internal audit programme shall address all elements of the management system, including the testing and/or calibration activities. It is the responsibility of the quality manager to plan and organize audits as required by the schedule and requested by management. Such audits shall be carried out by trained and qualified personnel who are, wherever resources permit, independent of the activity to be audited.

NOTE The cycle for internal auditing should normally be completed in one year.

4.14.2 When audit findings cast doubt on the effectiveness of the operations or on the correctness or validity of the laboratory's test or calibration results, the laboratory shall take timely corrective action, and shall notify customers in writing if investigations show that the laboratory results may have been affected.

4.14.3 The area of activity audited, the audit findings and corrective actions that arise from them shall be recorded.

4.14.4 Follow-up audit activities shall verify and record the implementation and effectiveness of the corrective action taken.

4.15 Management reviews

4.15.1 In accordance with a predetermined schedule and procedure, the laboratory's top management shall periodically conduct a review of the laboratory's management system and testing and/or calibration activities to ensure their continuing suitability and effectiveness, and to introduce necessary changes or improvements. The review shall take account of:

- the suitability of policies and procedures;
- reports from managerial and supervisory personnel;
- the outcome of recent internal audits;
- corrective and preventive actions;
- assessments by external bodies;
- the results of interlaboratory comparisons or proficiency tests;
- changes in the volume and type of the work;
- customer feedback;
- complaints;
- recommendations for improvement;
- other relevant factors, such as quality control activities, resources and staff training.

NOTE 1 A typical period for conducting a management review is once every 12 months.

NOTE 2 Results should feed into the laboratory planning system and should include the goals, objectives and action plans for the coming year.

NOTE 3 A management review includes consideration of related subjects at regular management meetings.

4.15.2 Findings from management reviews and the actions that arise from them shall be recorded. The management shall ensure that those actions are carried out within an appropriate and agreed timescale.

5 Technical requirements

5.1 General

5.1.1 Many factors determine the correctness and reliability of the tests and/or calibrations performed by a laboratory. These factors include contributions from:

- human factors (5.2);

- accommodation and environmental conditions (5.3);
- test and calibration methods and method validation (5.4);
- equipment (5.5);
- measurement traceability (5.6);
- sampling (5.7);
- the handling of test and calibration items (5.8).

5.1.2 The extent to which the factors contribute to the total uncertainty of measurement differs considerably between (types of) tests and between (types of) calibrations. The laboratory shall take account of these factors in developing test and calibration methods and procedures, in the training and qualification of personnel, and in the selection and calibration of the equipment it uses.

5.2 Personnel

5.2.1 The laboratory management shall ensure the competence of all who operate specific equipment, perform tests and/or calibrations, evaluate results, and sign test reports and calibration certificates. When using staff who are undergoing training, appropriate supervision shall be provided. Personnel performing specific tasks shall be qualified on the basis of appropriate education, training, experience and/or demonstrated skills, as required.

NOTE 1 In some technical areas (e.g. non-destructive testing) it may be required that the personnel performing certain tasks hold personnel certification. The laboratory is responsible for fulfilling specified personnel certification requirements. The requirements for personnel certification might be regulatory, included in the standards for the specific technical field, or required by the customer.

NOTE 2 The personnel responsible for the opinions and interpretation included in test reports should, in addition to the appropriate qualifications, training, experience and satisfactory knowledge of the testing carried out, also have:

- relevant knowledge of the technology used for the manufacturing of the items, materials, products, etc. tested, or the way they are used or intended to be used, and of the defects or degradations which may occur during or in service;
- knowledge of the general requirements expressed in the legislation and standards; and
- an understanding of the significance of deviations found with regard to the normal use of the items, materials, products, etc. concerned.

5.2.2 The management of the laboratory shall formulate the goals with respect to the education, training and skills of the laboratory personnel. The laboratory shall have a policy and procedures for identifying training needs and providing training of personnel. The training programme shall be relevant to the present and anticipated tasks of the laboratory. The effectiveness of the training actions taken shall be evaluated.

5.2.3 The laboratory shall use personnel who are employed by, or under contract to, the laboratory. Where contracted and additional technical and key support personnel are used, the laboratory shall ensure that such personnel are supervised and competent and that they work in accordance with the laboratory's management system.

5.2.4 The laboratory shall maintain current job descriptions for managerial, technical and key support personnel involved in tests and/or calibrations.

NOTE Job descriptions can be defined in many ways. As a minimum, the following should be defined:

- the responsibilities with respect to performing tests and/or calibrations;
- the responsibilities with respect to the planning of tests and/or calibrations and evaluation of results;
- the responsibilities for reporting opinions and interpretations;
- the responsibilities with respect to method modification and development and validation of new methods;

- expertise and experience required;
- qualifications and training programmes;
- managerial duties.

5.2.5 The management shall authorize specific personnel to perform particular types of sampling, test and/or calibration, to issue test reports and calibration certificates, to give opinions and interpretations and to operate particular types of equipment. The laboratory shall maintain records of the relevant authorization(s), competence, educational and professional qualifications, training, skills and experience of all technical personnel, including contracted personnel. This information shall be readily available and shall include the date on which authorization and/or competence is confirmed.

5.3 Accommodation and environmental conditions

5.3.1 Laboratory facilities for testing and/or calibration, including but not limited to energy sources, lighting and environmental conditions, shall be such as to facilitate correct performance of the tests and/or calibrations.

The laboratory shall ensure that the environmental conditions do not invalidate the results or adversely affect the required quality of any measurement. Particular care shall be taken when sampling and tests and/or calibrations are undertaken at sites other than a permanent laboratory facility. The technical requirements for accommodation and environmental conditions that can affect the results of tests and calibrations shall be documented.

5.3.2 The laboratory shall monitor, control and record environmental conditions as required by the relevant specifications, methods and procedures or where they influence the quality of the results. Due attention shall be paid, for example, to biological sterility, dust, electromagnetic disturbances, radiation, humidity, electrical supply, temperature, and sound and vibration levels, as appropriate to the technical activities concerned. Tests and calibrations shall be stopped when the environmental conditions jeopardize the results of the tests and/or calibrations.

5.3.3 There shall be effective separation between neighbouring areas in which there are incompatible activities. Measures shall be taken to prevent cross-contamination.

5.3.4 Access to and use of areas affecting the quality of the tests and/or calibrations shall be controlled. The laboratory shall determine the extent of control based on its particular circumstances.

5.3.5 Measures shall be taken to ensure good housekeeping in the laboratory. Special procedures shall be prepared where necessary.

5.4 Test and calibration methods and method validation

5.4.1 General

The laboratory shall use appropriate methods and procedures for all tests and/or calibrations within its scope. These include sampling, handling, transport, storage and preparation of items to be tested and/or calibrated, and, where appropriate, an estimation of the measurement uncertainty as well as statistical techniques for analysis of test and/or calibration data.

The laboratory shall have instructions on the use and operation of all relevant equipment, and on the handling and preparation of items for testing and/or calibration, or both, where the absence of such instructions could jeopardize the results of tests and/or calibrations. All instructions, standards, manuals and reference data relevant to the work of the laboratory shall be kept up to date and shall be made readily available to personnel (see 4.3). Deviation from test and calibration methods shall occur only if the deviation has been documented, technically justified, authorized, and accepted by the customer.

NOTE International, regional or national standards or other recognized specifications that contain sufficient and concise information on how to perform the tests and/or calibrations do not need to be supplemented or rewritten as internal procedures if these standards are written in a way that they can be used as published by the operating staff in a laboratory. It may be necessary to provide additional documentation for optional steps in the method or additional details.

5.4.2 Selection of methods

The laboratory shall use test and/or calibration methods, including methods for sampling, which meet the needs of the customer and which are appropriate for the tests and/or calibrations it undertakes. Methods published in international, regional or national standards shall preferably be used. The laboratory shall ensure that it uses the latest valid edition of a standard unless it is not appropriate or possible to do so. When necessary, the standard shall be supplemented with additional details to ensure consistent application.

When the customer does not specify the method to be used, the laboratory shall select appropriate methods that have been published either in international, regional or national standards, or by reputable technical organizations, or in relevant scientific texts or journals, or as specified by the manufacturer of the equipment. Laboratory-developed methods or methods adopted by the laboratory may also be used if they are appropriate for the intended use and if they are validated. The customer shall be informed as to the method chosen. The laboratory shall confirm that it can properly operate standard methods before introducing the tests or calibrations. If the standard method changes, the confirmation shall be repeated.

The laboratory shall inform the customer when the method proposed by the customer is considered to be inappropriate or out of date.

5.4.3 Laboratory-developed methods

The introduction of test and calibration methods developed by the laboratory for its own use shall be a planned activity and shall be assigned to qualified personnel equipped with adequate resources.

Plans shall be updated as development proceeds and effective communication amongst all personnel involved shall be ensured.

5.4.4 Non-standard methods

When it is necessary to use methods not covered by standard methods, these shall be subject to agreement with the customer and shall include a clear specification of the customer's requirements and the purpose of the test and/or calibration. The method developed shall have been validated appropriately before use.

NOTE For new test and/or calibration methods, procedures should be developed prior to the tests and/or calibrations being performed and should contain at least the following information:

- a) appropriate identification;
- b) scope;
- c) description of the type of item to be tested or calibrated;
- d) parameters or quantities and ranges to be determined;
- e) apparatus and equipment, including technical performance requirements;
- f) reference standards and reference materials required;
- g) environmental conditions required and any stabilization period needed;
- h) description of the procedure, including
 - affixing of identification marks, handling, transporting, storing and preparation of items,
 - checks to be made before the work is started,
 - checks that the equipment is working properly and, where required, calibration and adjustment of the equipment before each use,
 - the method of recording the observations and results,
 - any safety measures to be observed;
- i) criteria and/or requirements for approval/rejection;
- j) data to be recorded and method of analysis and presentation;
- k) the uncertainty or the procedure for estimating uncertainty.

5.4.5 Validation of methods

5.4.5.1 Validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled.

5.4.5.2 The laboratory shall validate non-standard methods, laboratory-designed/developed methods, standard methods used outside their intended scope, and amplifications and modifications of standard methods to confirm that the methods are fit for the intended use. The validation shall be as extensive as is necessary to meet the needs of the given application or field of application. The laboratory shall record the results obtained, the procedure used for the validation, and a statement as to whether the method is fit for the intended use.

NOTE 1 Validation may include procedures for sampling, handling and transportation.

NOTE 2 The techniques used for the determination of the performance of a method should be one of, or a combination of, the following:

- calibration using reference standards or reference materials;
- comparison of results achieved with other methods;
- interlaboratory comparisons;
- systematic assessment of the factors influencing the result;
- assessment of the uncertainty of the results based on scientific understanding of the theoretical principles of the method and practical experience.

NOTE 3 When some changes are made in the validated non-standard methods, the influence of such changes should be documented and, if appropriate, a new validation should be carried out.

5.4.5.3 The range and accuracy of the values obtainable from validated methods (e.g. the uncertainty of the results, detection limit, selectivity of the method, linearity, limit of repeatability and/or reproducibility, robustness against external influences and/or cross-sensitivity against interference from the matrix of the sample/test object), as assessed for the intended use, shall be relevant to the customers' needs.

NOTE 1 Validation includes specification of the requirements, determination of the characteristics of the methods, a check that the requirements can be fulfilled by using the method, and a statement on the validity.

NOTE 2 As method-development proceeds, regular review should be carried out to verify that the needs of the customer are still being fulfilled. Any change in requirements requiring modifications to the development plan should be approved and authorized.

NOTE 3 Validation is always a balance between costs, risks and technical possibilities. There are many cases in which the range and uncertainty of the values (e.g. accuracy, detection limit, selectivity, linearity, repeatability, reproducibility, robustness and cross-sensitivity) can only be given in a simplified way due to lack of information.

5.4.6 Estimation of uncertainty of measurement

5.4.6.1 A calibration laboratory, or a testing laboratory performing its own calibrations, shall have and shall apply a procedure to estimate the uncertainty of measurement for all calibrations and types of calibrations.

5.4.6.2 Testing laboratories shall have and shall apply procedures for estimating uncertainty of measurement. In certain cases the nature of the test method may preclude rigorous, metrologically and statistically valid, calculation of uncertainty of measurement. In these cases the laboratory shall at least attempt to identify all the components of uncertainty and make a reasonable estimation, and shall ensure that the form of reporting of the result does not give a wrong impression of the uncertainty. Reasonable estimation shall be based on knowledge of the performance of the method and on the measurement scope and shall make use of, for example, previous experience and validation data.

NOTE 1 The degree of rigor needed in an estimation of uncertainty of measurement depends on factors such as:

- the requirements of the test method;

- the requirements of the customer;
- the existence of narrow limits on which decisions on conformity to a specification are based.

NOTE 2 In those cases where a well-recognized test method specifies limits to the values of the major sources of uncertainty of measurement and specifies the form of presentation of calculated results, the laboratory is considered to have satisfied this clause by following the test method and reporting instructions (see 5.10).

5.4.6.3 When estimating the uncertainty of measurement, all uncertainty components which are of importance in the given situation shall be taken into account using appropriate methods of analysis.

NOTE 1 Sources contributing to the uncertainty include, but are not necessarily limited to, the reference standards and reference materials used, methods and equipment used, environmental conditions, properties and condition of the item being tested or calibrated, and the operator.

NOTE 2 The predicted long-term behaviour of the tested and/or calibrated item is not normally taken into account when estimating the measurement uncertainty.

NOTE 3 For further information, see ISO 5725 and the Guide to the Expression of Uncertainty in Measurement (see Bibliography).

5.4.7 Control of data

5.4.7.1 Calculations and data transfers shall be subject to appropriate checks in a systematic manner.

5.4.7.2 When computers or automated equipment are used for the acquisition, processing, recording, reporting, storage or retrieval of test or calibration data, the laboratory shall ensure that:

- a) computer software developed by the user is documented in sufficient detail and is suitably validated as being adequate for use;
- b) procedures are established and implemented for protecting the data; such procedures shall include, but not be limited to, integrity and confidentiality of data entry or collection, data storage, data transmission and data processing;
- c) computers and automated equipment are maintained to ensure proper functioning and are provided with the environmental and operating conditions necessary to maintain the integrity of test and calibration data.

NOTE Commercial off-the-shelf software (e.g. wordprocessing, database and statistical programmes) in general use within their designed application range may be considered to be sufficiently validated. However, laboratory software configuration/modifications should be validated as in 5.4.7.2 a).

5.5 Equipment

5.5.1 The laboratory shall be furnished with all items of sampling, measurement and test equipment required for the correct performance of the tests and/or calibrations (including sampling, preparation of test and/or calibration items, processing and analysis of test and/or calibration data). In those cases where the laboratory needs to use equipment outside its permanent control, it shall ensure that the requirements of this International Standard are met.

5.5.2 Equipment and its software used for testing, calibration and sampling shall be capable of achieving the accuracy required and shall comply with specifications relevant to the tests and/or calibrations concerned. Calibration programmes shall be established for key quantities or values of the instruments where these properties have a significant effect on the results. Before being placed into service, equipment (including that used for sampling) shall be calibrated or checked to establish that it meets the laboratory's specification requirements and complies with the relevant standard specifications. It shall be checked and/or calibrated before use (see 5.6).

5.5.3 Equipment shall be operated by authorized personnel. Up-to-date instructions on the use and maintenance of equipment (including any relevant manuals provided by the manufacturer of the equipment) shall be readily available for use by the appropriate laboratory personnel.

5.5.4 Each item of equipment and its software used for testing and calibration and significant to the result shall, when practicable, be uniquely identified.

5.5.5 Records shall be maintained of each item of equipment and its software significant to the tests and/or calibrations performed. The records shall include at least the following:

- a) the identity of the item of equipment and its software;
- b) the manufacturer's name, type identification, and serial number or other unique identification;
- c) checks that equipment complies with the specification (see 5.5.2);
- d) the current location, where appropriate;
- e) the manufacturer's instructions, if available, or reference to their location;
- f) dates, results and copies of reports and certificates of all calibrations, adjustments, acceptance criteria, and the due date of next calibration;
- g) the maintenance plan, where appropriate, and maintenance carried out to date;
- h) any damage, malfunction, modification or repair to the equipment.

5.5.6 The laboratory shall have procedures for safe handling, transport, storage, use and planned maintenance of measuring equipment to ensure proper functioning and in order to prevent contamination or deterioration.

NOTE Additional procedures may be necessary when measuring equipment is used outside the permanent laboratory for tests, calibrations or sampling.

5.5.7 Equipment that has been subjected to overloading or mishandling, gives suspect results, or has been shown to be defective or outside specified limits, shall be taken out of service. It shall be isolated to prevent its use or clearly labelled or marked as being out of service until it has been repaired and shown by calibration or test to perform correctly. The laboratory shall examine the effect of the defect or departure from specified limits on previous tests and/or calibrations and shall institute the "Control of nonconforming work" procedure (see 4.9).

5.5.8 Whenever practicable, all equipment under the control of the laboratory and requiring calibration shall be labelled, coded or otherwise identified to indicate the status of calibration, including the date when last calibrated and the date or expiration criteria when recalibration is due.

5.5.9 When, for whatever reason, equipment goes outside the direct control of the laboratory, the laboratory shall ensure that the function and calibration status of the equipment are checked and shown to be satisfactory before the equipment is returned to service.

5.5.10 When intermediate checks are needed to maintain confidence in the calibration status of the equipment, these checks shall be carried out according to a defined procedure.

5.5.11 Where calibrations give rise to a set of correction factors, the laboratory shall have procedures to ensure that copies (e.g. in computer software) are correctly updated.

5.5.12 Test and calibration equipment, including both hardware and software, shall be safeguarded from adjustments which would invalidate the test and/or calibration results.

5.6 Measurement traceability

5.6.1 General

All equipment used for tests and/or calibrations, including equipment for subsidiary measurements (e.g. for environmental conditions) having a significant effect on the accuracy or validity of the result of the test, calibration or sampling shall be calibrated before being put into service. The laboratory shall have an established programme and procedure for the calibration of its equipment.

NOTE Such a programme should include a system for selecting, using, calibrating, checking, controlling and maintaining measurement standards, reference materials used as measurement standards, and measuring and test equipment used to perform tests and calibrations.

5.6.2 Specific requirements

5.6.2.1 Calibration

5.6.2.1.1 For calibration laboratories, the programme for calibration of equipment shall be designed and operated so as to ensure that calibrations and measurements made by the laboratory are traceable to the International System of Units (SI) (*Système international d'unités*).

A calibration laboratory establishes traceability of its own measurement standards and measuring instruments to the SI by means of an unbroken chain of calibrations or comparisons linking them to relevant primary standards of the SI units of measurement. The link to SI units may be achieved by reference to national measurement standards. National measurement standards may be primary standards, which are primary realizations of the SI units or agreed representations of SI units based on fundamental physical constants, or they may be secondary standards which are standards calibrated by another national metrology institute. When using external calibration services, traceability of measurement shall be assured by the use of calibration services from laboratories that can demonstrate competence, measurement capability and traceability. The calibration certificates issued by these laboratories shall contain the measurement results, including the measurement uncertainty and/or a statement of compliance with an identified metrological specification (see also 5.10.4.2).

NOTE 1 Calibration laboratories fulfilling the requirements of this International Standard are considered to be competent. A calibration certificate bearing an accreditation body logo from a calibration laboratory accredited to this International Standard, for the calibration concerned, is sufficient evidence of traceability of the calibration data reported.

NOTE 2 Traceability to SI units of measurement may be achieved by reference to an appropriate primary standard (see VIM:1993, 6.4) or by reference to a natural constant, the value of which in terms of the relevant SI unit is known and recommended by the General Conference of Weights and Measures (CGPM) and the International Committee for Weights and Measures (CIPM).

NOTE 3 Calibration laboratories that maintain their own primary standard or representation of SI units based on fundamental physical constants can claim traceability to the SI system only after these standards have been compared, directly or indirectly, with other similar standards of a national metrology institute.

NOTE 4 The term "identified metrological specification" means that it must be clear from the calibration certificate which specification the measurements have been compared with, by including the specification or by giving an unambiguous reference to the specification.

NOTE 5 When the terms "international standard" or "national standard" are used in connection with traceability, it is assumed that these standards fulfil the properties of primary standards for the realization of SI units.

NOTE 6 Traceability to national measurement standards does not necessarily require the use of the national metrology institute of the country in which the laboratory is located.

NOTE 7 If a calibration laboratory wishes or needs to obtain traceability from a national metrology institute other than in its own country, this laboratory should select a national metrology institute that actively participates in the activities of BIPM either directly or through regional groups.

NOTE 8 The unbroken chain of calibrations or comparisons may be achieved in several steps carried out by different laboratories that can demonstrate traceability.

5.6.2.1.2 There are certain calibrations that currently cannot be strictly made in SI units. In these cases calibration shall provide confidence in measurements by establishing traceability to appropriate measurement standards such as:

- the use of certified reference materials provided by a competent supplier to give a reliable physical or chemical characterization of a material;
- the use of specified methods and/or consensus standards that are clearly described and agreed by all parties concerned.

Participation in a suitable programme of interlaboratory comparisons is required where possible.

5.6.2.2 Testing

5.6.2.2.1 For testing laboratories, the requirements given in 5.6.2.1 apply for measuring and test equipment with measuring functions used, unless it has been established that the associated contribution from the calibration contributes little to the total uncertainty of the test result. When this situation arises, the laboratory shall ensure that the equipment used can provide the uncertainty of measurement needed.

NOTE The extent to which the requirements in 5.6.2.1 should be followed depends on the relative contribution of the calibration uncertainty to the total uncertainty. If calibration is the dominant factor, the requirements should be strictly followed.

5.6.2.2.2 Where traceability of measurements to SI units is not possible and/or not relevant, the same requirements for traceability to, for example, certified reference materials, agreed methods and/or consensus standards, are required as for calibration laboratories (see 5.6.2.1.2).

5.6.3 Reference standards and reference materials

5.6.3.1 Reference standards

The laboratory shall have a programme and procedure for the calibration of its reference standards. Reference standards shall be calibrated by a body that can provide traceability as described in 5.6.2.1. Such reference standards of measurement held by the laboratory shall be used for calibration only and for no other purpose, unless it can be shown that their performance as reference standards would not be invalidated. Reference standards shall be calibrated before and after any adjustment.

5.6.3.2 Reference materials

Reference materials shall, where possible, be traceable to SI units of measurement, or to certified reference materials. Internal reference materials shall be checked as far as is technically and economically practicable.

5.6.3.3 Intermediate checks

Checks needed to maintain confidence in the calibration status of reference, primary, transfer or working standards and reference materials shall be carried out according to defined procedures and schedules.

5.6.3.4 Transport and storage

The laboratory shall have procedures for safe handling, transport, storage and use of reference standards and reference materials in order to prevent contamination or deterioration and in order to protect their integrity.

NOTE Additional procedures may be necessary when reference standards and reference materials are used outside the permanent laboratory for tests, calibrations or sampling.

5.7 Sampling

5.7.1 The laboratory shall have a sampling plan and procedures for sampling when it carries out sampling of substances, materials or products for subsequent testing or calibration. The sampling plan as well as the sampling procedure shall be available at the location where sampling is undertaken. Sampling plans shall, whenever reasonable, be based on appropriate statistical methods. The sampling process shall address the factors to be controlled to ensure the validity of the test and calibration results.

NOTE 1 Sampling is a defined procedure whereby a part of a substance, material or product is taken to provide for testing or calibration of a representative sample of the whole. Sampling may also be required by the appropriate specification for which the substance, material or product is to be tested or calibrated. In certain cases (e.g. forensic analysis), the sample may not be representative but is determined by availability.

NOTE 2 Sampling procedures should describe the selection, sampling plan, withdrawal and preparation of a sample or samples from a substance, material or product to yield the required information.

5.7.2 Where the customer requires deviations, additions or exclusions from the documented sampling procedure, these shall be recorded in detail with the appropriate sampling data and shall be included in all documents containing test and/or calibration results, and shall be communicated to the appropriate personnel.

5.7.3 The laboratory shall have procedures for recording relevant data and operations relating to sampling that forms part of the testing or calibration that is undertaken. These records shall include the sampling procedure used, the identification of the sampler, environmental conditions (if relevant) and diagrams or other equivalent means to identify the sampling location as necessary and, if appropriate, the statistics the sampling procedures are based upon.

5.8 Handling of test and calibration items

5.8.1 The laboratory shall have procedures for the transportation, receipt, handling, protection, storage, retention and/or disposal of test and/or calibration items, including all provisions necessary to protect the integrity of the test or calibration item, and to protect the interests of the laboratory and the customer.

5.8.2 The laboratory shall have a system for identifying test and/or calibration items. The identification shall be retained throughout the life of the item in the laboratory. The system shall be designed and operated so as to ensure that items cannot be confused physically or when referred to in records or other documents. The system shall, if appropriate, accommodate a sub-division of groups of items and the transfer of items within and from the laboratory.

5.8.3 Upon receipt of the test or calibration item, abnormalities or departures from normal or specified conditions, as described in the test or calibration method, shall be recorded. When there is doubt as to the suitability of an item for test or calibration, or when an item does not conform to the description provided, or the test or calibration required is not specified in sufficient detail, the laboratory shall consult the customer for further instructions before proceeding and shall record the discussion.

5.8.4 The laboratory shall have procedures and appropriate facilities for avoiding deterioration, loss or damage to the test or calibration item during storage, handling and preparation. Handling instructions provided with the item shall be followed. When items have to be stored or conditioned under specified environmental conditions, these conditions shall be maintained, monitored and recorded. Where a test or calibration item or a portion of an item is to be held secure, the laboratory shall have arrangements for storage and security that protect the condition and integrity of the secured items or portions concerned.

NOTE 1 Where test items are to be returned into service after testing, special care is required to ensure that they are not damaged or injured during the handling, testing or storing/waiting processes.

NOTE 2 A sampling procedure and information on storage and transport of samples, including information on sampling factors influencing the test or calibration result, should be provided to those responsible for taking and transporting the samples.

NOTE 3 Reasons for keeping a test or calibration item secure can be for reasons of record, safety or value, or to enable complementary tests and/or calibrations to be performed later.

5.9 Assuring the quality of test and calibration results

5.9.1 The laboratory shall have quality control procedures for monitoring the validity of tests and calibrations undertaken. The resulting data shall be recorded in such a way that trends are detectable and, where practicable, statistical techniques shall be applied to the reviewing of the results. This monitoring shall be planned and reviewed and may include, but not be limited to, the following:

- a) regular use of certified reference materials and/or internal quality control using secondary reference materials;
- b) participation in interlaboratory comparison or proficiency-testing programmes;
- c) replicate tests or calibrations using the same or different methods;
- d) retesting or recalibration of retained items;
- e) correlation of results for different characteristics of an item.

NOTE The selected methods should be appropriate for the type and volume of the work undertaken.

5.9.2 Quality control data shall be analysed and, where they are found to be outside pre-defined criteria, planned action shall be taken to correct the problem and to prevent incorrect results from being reported.

5.10 Reporting the results

5.10.1 General

The results of each test, calibration, or series of tests or calibrations carried out by the laboratory shall be reported accurately, clearly, unambiguously and objectively, and in accordance with any specific instructions in the test or calibration methods.

The results shall be reported, usually in a test report or a calibration certificate (see Note 1), and shall include all the information requested by the customer and necessary for the interpretation of the test or calibration results and all information required by the method used. This information is normally that required by 5.10.2, and 5.10.3 or 5.10.4.

In the case of tests or calibrations performed for internal customers, or in the case of a written agreement with the customer, the results may be reported in a simplified way. Any information listed in 5.10.2 to 5.10.4 which is not reported to the customer shall be readily available in the laboratory which carried out the tests and/or calibrations.

NOTE 1 Test reports and calibration certificates are sometimes called test certificates and calibration reports, respectively.

NOTE 2 The test reports or calibration certificates may be issued as hard copy or by electronic data transfer provided that the requirements of this International Standard are met.

5.10.2 Test reports and calibration certificates

Each test report or calibration certificate shall include at least the following information, unless the laboratory has valid reasons for not doing so:

- a) a title (e.g. "Test Report" or "Calibration Certificate");
- b) the name and address of the laboratory, and the location where the tests and/or calibrations were carried out, if different from the address of the laboratory;

- c) unique identification of the test report or calibration certificate (such as the serial number), and on each page an identification in order to ensure that the page is recognized as a part of the test report or calibration certificate, and a clear identification of the end of the test report or calibration certificate;
- d) the name and address of the customer;
- e) identification of the method used;
- f) a description of, the condition of, and unambiguous identification of the item(s) tested or calibrated;
- g) the date of receipt of the test or calibration item(s) where this is critical to the validity and application of the results, and the date(s) of performance of the test or calibration;
- h) reference to the sampling plan and procedures used by the laboratory or other bodies where these are relevant to the validity or application of the results;
- i) the test or calibration results with, where appropriate, the units of measurement;
- j) the name(s), function(s) and signature(s) or equivalent identification of person(s) authorizing the test report or calibration certificate;
- k) where relevant, a statement to the effect that the results relate only to the items tested or calibrated.

NOTE 1 Hard copies of test reports and calibration certificates should also include the page number and total number of pages.

NOTE 2 It is recommended that laboratories include a statement specifying that the test report or calibration certificate shall not be reproduced except in full, without written approval of the laboratory.

5.10.3 Test reports

5.10.3.1 In addition to the requirements listed in 5.10.2, test reports shall, where necessary for the interpretation of the test results, include the following:

- a) deviations from, additions to, or exclusions from the test method, and information on specific test conditions, such as environmental conditions;
- b) where relevant, a statement of compliance/non-compliance with requirements and/or specifications;
- c) where applicable, a statement on the estimated uncertainty of measurement; information on uncertainty is needed in test reports when it is relevant to the validity or application of the test results, when a customer's instruction so requires, or when the uncertainty affects compliance to a specification limit;
- d) where appropriate and needed, opinions and interpretations (see 5.10.5);
- e) additional information which may be required by specific methods, customers or groups of customers.

5.10.3.2 In addition to the requirements listed in 5.10.2 and 5.10.3.1, test reports containing the results of sampling shall include the following, where necessary for the interpretation of test results:

- a) the date of sampling;
- b) unambiguous identification of the substance, material or product sampled (including the name of the manufacturer, the model or type of designation and serial numbers as appropriate);
- c) the location of sampling, including any diagrams, sketches or photographs;
- d) a reference to the sampling plan and procedures used;

- e) details of any environmental conditions during sampling that may affect the interpretation of the test results;
- f) any standard or other specification for the sampling method or procedure, and deviations, additions to or exclusions from the specification concerned.

5.10.4 Calibration certificates

5.10.4.1 In addition to the requirements listed in 5.10.2, calibration certificates shall include the following, where necessary for the interpretation of calibration results:

- a) the conditions (e.g. environmental) under which the calibrations were made that have an influence on the measurement results;
- b) the uncertainty of measurement and/or a statement of compliance with an identified metrological specification or clauses thereof;
- c) evidence that the measurements are traceable (see Note 2 in 5.6.2.1.1).

5.10.4.2 The calibration certificate shall relate only to quantities and the results of functional tests. If a statement of compliance with a specification is made, this shall identify which clauses of the specification are met or not met.

When a statement of compliance with a specification is made omitting the measurement results and associated uncertainties, the laboratory shall record those results and maintain them for possible future reference.

When statements of compliance are made, the uncertainty of measurement shall be taken into account.

5.10.4.3 When an instrument for calibration has been adjusted or repaired, the calibration results before and after adjustment or repair, if available, shall be reported.

5.10.4.4 A calibration certificate (or calibration label) shall not contain any recommendation on the calibration interval except where this has been agreed with the customer. This requirement may be superseded by legal regulations.

5.10.5 Opinions and interpretations

When opinions and interpretations are included, the laboratory shall document the basis upon which the opinions and interpretations have been made. Opinions and interpretations shall be clearly marked as such in a test report.

NOTE 1 Opinions and interpretations should not be confused with inspections and product certifications as intended in ISO/IEC 17020 and ISO/IEC Guide 65.

NOTE 2 Opinions and interpretations included in a test report may comprise, but not be limited to, the following:

- an opinion on the statement of compliance/noncompliance of the results with requirements;
- fulfilment of contractual requirements;
- recommendations on how to use the results;
- guidance to be used for improvements.

NOTE 3 In many cases it might be appropriate to communicate the opinions and interpretations by direct dialogue with the customer. Such dialogue should be written down.

5.10.6 Testing and calibration results obtained from subcontractors

When the test report contains results of tests performed by subcontractors, these results shall be clearly identified. The subcontractor shall report the results in writing or electronically.

When a calibration has been subcontracted, the laboratory performing the work shall issue the calibration certificate to the contracting laboratory.

5.10.7 Electronic transmission of results

In the case of transmission of test or calibration results by telephone, telex, facsimile or other electronic or electromagnetic means, the requirements of this International Standard shall be met (see also 5.4.7).

5.10.8 Format of reports and certificates

The format shall be designed to accommodate each type of test or calibration carried out and to minimize the possibility of misunderstanding or misuse.

NOTE 1 Attention should be given to the lay-out of the test report or calibration certificate, especially with regard to the presentation of the test or calibration data and ease of assimilation by the reader.

NOTE 2 The headings should be standardized as far as possible.

5.10.9 Amendments to test reports and calibration certificates

Material amendments to a test report or calibration certificate after issue shall be made only in the form of a further document, or data transfer, which includes the statement:

"Supplement to Test Report [or Calibration Certificate], serial number... [or as otherwise identified]",

or an equivalent form of wording.

Such amendments shall meet all the requirements of this International Standard.

When it is necessary to issue a complete new test report or calibration certificate, this shall be uniquely identified and shall contain a reference to the original that it replaces.

Annex A (informative)

Nominal cross-references to ISO 9001:2000

Table A.1 — Nominal cross-references to ISO 9001:2000

| ISO 9001:2000 | ISO/IEC 17025 |
|---------------|---|
| Clause 1 | Clause 1 |
| Clause 2 | Clause 2 |
| Clause 3 | Clause 3 |
| | |
| 4.1 | 4.1, 4.1.1, 4.1.2, 4.1.3, 4.1.4, 4.1.5, 4.2, 4.2.1, 4.2.2, 4.2.3, 4.2.4 |
| 4.2.1 | 4.2.2, 4.2.3, 4.3.1 |
| 4.2.2 | 4.2.2, 4.2.3, 4.2.4 |
| 4.2.3 | 4.3 |
| 4.2.4 | 4.3.1, 4.12 |
| | |
| 5.1 | 4.2.2, 4.2.3 |
| 5.1 a) | 4.1.2, 4.1.6 |
| 5.1 b) | 4.2.2 |
| 5.1 c) | 4.2.2 |
| 5.1 d) | 4.15 |
| 5.1 e) | 4.1.5 |
| 5.2 | 4.4.1 |
| 5.3 | 4.2.2 |
| 5.3 a) | 4.2.2 |
| 5.3 b) | 4.2.3 |
| 5.3 c) | 4.2.2 |
| 5.3 d) | 4.2.2 |
| 5.3 e) | 4.2.2 |
| 5.4.1 | 4.2.2 c) |
| 5.4.2 | 4.2.1 |
| 5.4.2 a) | 4.2.1 |
| 5.4.2 b) | 4.2.1 |
| 5.5.1 | 4.1.5 a), f), h) |
| 5.5.2 | 4.1.5 i) |
| 5.5.2 a) | 4.1.5 i) |
| 5.5.2 b) | 4.11.1 |
| 5.5.2 c) | 4.2.4 |
| 5.5.3 | 4.1.6 |
| 5.6.1 | 4.15 |
| 5.6.2 | 4.15 |
| 5.6.3 | 4.15 |

| ISO 9001:2000 | ISO/IEC 17025 |
|---------------|---|
| | |
| 6.1 a) | 4.10 |
| 6.1 b) | 4.4.1, 4.7, 5.4.2, 5.4.3, 5.4.4, 5.10.1 |
| 6.2.1 | 5.2.1 |
| 6.2.2 a) | 5.2.2, 5.5.3 |
| 6.2.2 b) | 5.2.1, 5.2.2 |
| 6.2.2 c) | 5.2.2 |
| 6.2.2 d) | 4.1.5 k) |
| 6.2.2 e) | 5.2.5 |
| 6.3.1 a) | 4.1.3, 4.12.1.2, 4.12.1.3, 5.3 |
| 6.3.1 b) | 4.12.1.4, 5.4.7.2, 5.5, 5.6 |
| 6.3.1 c) | 4.6, 5.5.6, 5.6.3.4, 5.8, 5.10 |
| 6.4 | 5.3.1, 5.3.2, 5.3.3, 5.3.4, 5.3.5 |
| | |
| 7.1 | 5.1 |
| 7.1 a) | 4.2.2 |
| 7.1 b) | 4.1.5 a), 4.2.1, 4.2.3 |
| 7.1 c) | 5.4, 5.9 |
| 7.1 d) | 4.1, 5.4, 5.9 |
| 7.2.1 | 4.4.1, 4.4.2, 4.4.3, 4.4.4, 4.4.5, 5.4, 5.9, 5.10 |
| 7.2.2 | 4.4.1, 4.4.2, 4.4.3, 4.4.4, 4.4.5, 5.4, 5.9, 5.10 |
| 7.2.3 | 4.4.2, 4.4.4, 4.5, 4.7, 4.8 |
| 7.3 | 5, 5.4, 5.9 |
| 7.4.1 | 4.6.1, 4.6.2, 4.6.4 |
| 7.4.2 | 4.6.3 |
| 7.4.3 | 4.6.2 |
| 7.5.1 | 5.1, 5.2, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9 |
| 7.5.2 | 5.2.5, 5.4.2, 5.4.5 |
| 7.5.3 | 5.8.2 |
| 7.5.4 | 4.1.5 c), 5.8 |
| 7.5.5 | 4.6.1, 4.12, 5.8, 5.10 |
| 7.6 | 5.4, 5.5 |
| | |
| 8.1 | 4.10, 5.4, 5.9 |
| 8.2.1 | 4.10 |
| 8.2.2 | 4.11.5, 4.14 |
| 8.2.3 | 4.11.5, 4.14, 5.9 |
| 8.2.4 | 4.5, 4.6, 4.9, 5.5.2, 5.5.9, 5.8, 5.8.3, 5.8.4, 5.9 |
| 8.3 | 4.9 |
| 8.4 | 4.10, 5.9 |
| 8.5.1 | 4.10, 4.12 |
| 8.5.2 | 4.11, 4.12 |
| 8.5.3 | 4.9, 4.11, 4.12 |

ISO/IEC 17025 covers several technical competence requirements that are not covered by ISO 9001:2000.

Annex B

(informative)

Guidelines for establishing applications for specific fields

B.1 The requirements specified in this International Standard are stated in general terms and, while they are applicable to all test and calibration laboratories, explanations might be needed. Such explanations on applications are herein referred to as applications. Applications should not include additional general requirements not included in this International Standard.

B.2 Applications can be thought of as an elaboration of the generally stated criteria (requirements) of this International Standard for specified fields of test and calibration, test technologies, products, materials or specific tests or calibrations. Accordingly, applications should be established by persons having appropriate technical knowledge and experience, and should address items that are essential or most important for the proper conduct of a test or calibration.

B.3 Depending on the application at hand, it may be necessary to establish applications for the technical requirements of this International Standard. Establishing applications may be accomplished by simply providing detail or adding extra information to the already generally stated requirements in each of the clauses (e.g. specific limitations to the temperature and humidity in the laboratory).

In some cases the applications will be quite limited, applying only to a given test or calibration method or to a group of calibration or test methods. In other cases the applications may be quite broad, applying to the testing or calibration of various products or items or to entire fields of testing or calibration.

B.4 If the applications apply to a group of test or calibration methods in an entire technical field, common wording should be used for all of the methods.

Alternatively, it may be necessary to develop a separate document of applications to supplement this International Standard for specific types or groups of tests or calibrations, products, materials or technical fields of tests or calibrations. Such a document should provide only the necessary supplementary information, while maintaining this International Standard as the governing document through reference. Applications which are too specific should be avoided in order to limit the proliferation of detailed documents.

B.5 The guidance in this annex should be used by accreditation bodies and other types of evaluation bodies when they develop applications for their own purposes (e.g. accreditation in specific areas).

Bibliography

- [1] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1 General principles and definitions*
- [2] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*
- [3] ISO 5725-3, *Accuracy (trueness and precision) of measurement methods and results — Part 3: Intermediate measures of the precision of a standard measurement method*
- [4] ISO 5725-4, *Accuracy (trueness and precision) of measurement methods and results — Part 4: Basic methods for the determination of the trueness of a standard measurement method*
- [5] ISO 5725-6, *Accuracy (trueness and precision) of measurement methods and results — Part 6: Use in practice of accuracy values*
- [6] ISO 9000:—¹⁾, *Quality management systems — Fundamentals and vocabulary*
- [7] ISO 9001:2000, *Quality management systems — Requirements*
- [8] ISO/IEC 90003, *Software engineering — Guidelines for the application of ISO 9001:2000 to computer software*
- [9] ISO 10012:2003, *Measurement management systems — Requirements for measurement processes and measuring equipment*
- [10] ISO/IEC 17011, *Conformity assessment — General requirements for accreditation bodies accrediting conformity assessment bodies*
- [11] ISO/IEC 17020, *General criteria for the operation of various types of bodies performing inspection*
- [12] ISO 19011, *Guidelines for quality and/or environmental management systems auditing*
- [13] ISO Guide 30, *Terms and definitions used in connection with reference materials*
- [14] ISO Guide 31, *Reference materials — Contents of certificates and labels*
- [15] ISO Guide 32, *Calibration in analytical chemistry and use of certified reference materials*
- [16] ISO Guide 33, *Uses of certified reference materials*
- [17] ISO Guide 34, *General requirements for the competence of reference material producers*
- [18] ISO Guide 35, *Certification of reference materials — General and statistical principles*
- [19] ISO/IEC Guide 43-1, *Proficiency testing by interlaboratory comparisons — Part 1: Development and operation of proficiency testing schemes*
- [20] ISO/IEC Guide 43-2, *Proficiency testing by interlaboratory comparisons — Part 2: Selection and use of proficiency testing schemes by laboratory accreditation bodies*

1) To be published. (Revision of ISO 9000:2000)

- [21] ISO/IEC Guide 58:1993, *Calibration and testing laboratory accreditation systems — General requirements for operation and recognition*
- [22] ISO/IEC Guide 65, *General requirements for bodies operating product certification systems*
- [23] GUM, *Guide to the Expression of Uncertainty in Measurement*, issued by BIPM, IEC, IFCC, ISO, IUPAC, IUPAP and OIML
- [24] Information and documents on laboratory accreditation can be found on the ILAC (International Laboratory Accreditation Cooperation): www.ilac.org

Ginnaven, Christopher C.

From: Michael Henson [m.henson@floydfairnessfund.org]
Sent: Thursday, April 26, 2007 12:00 PM
To: Suh, Maurice; Howard Jacobs; Paul Scott
Subject: Sport24 - Original French Version

Landis pris au piège

Selon L'Equipe, les analyses rétroactives effectuées sur sept échantillons de Floyd Landis démontrent bien la présence de testostérone exogène. L'Américain encourt deux ans de suspension.

Par Florian Egly

Des résultats accablants

Cette fois-ci, Floyd Landis ne pourra pas se retrancher derrière une prétendue production naturelle exceptionnellement élevée de testostérone ni se dérober en dénonçant la supposée incompétence du Laboratoire de Châtenay-Malabry (LNDD). Les deux lignes de défense avancées par le coureur américain depuis son contrôle positif sur le Tour de France sont ainsi tombées à plat. Selon une information de L'Equipe, sept échantillons appartenant à l'ancien Maillot Jaune et conservés depuis la Grande Boucle 2006 ont dévoilé la présence de testostérone exogène, lors d'analyses rétroactives réalisées la semaine dernière par le LNDD. Ces nouvelles pratiques avaient été commandées par l'USADA, l'Agence Américaine Antidopage, chargée du dossier Landis, sur la base d'une nouvelle technique plus fiable (l'IRMS), capable de différencier le taux de testostérone endogène d'une aide extérieure. Deux experts de l'agence, de même que deux représentants du coureur, ont assisté à ces analyses, ce qui révoque donc l'argument d'une soi-disant manipulation de la part du laboratoire français..

Pas une surprise

La surprise n'est pas immense. Ces révélations ne font que confirmer le contrôle positif de l'Américain au soir de sa «fantastique» chevauchée vers Morzine, le 20 juillet, et qui avait été conforté après examen de l'échantillon B quelques semaines plus tard. Seulement, Landis avait réussi jusqu'ici à s'en sortir par plusieurs pirouettes, multipliant tous les recours possibles et bénéficiant surtout de deux bourdes de procédure du Laboratoire de Châtenay-Malabry : une erreur d'étiquetage sur le procès-verbal de la contre-expertise et la présence de deux mêmes agents lors des contrôles des échantillons A et B, ce qui est interdit par le règlement. Convoqué par l'AFLD, l'Agence Française de Lutte contre le Dopage, en février dernier, l'ex-leader de Phonak avait négocié un report en acceptant de ne pas prendre le départ du Tour 2007, l'autorisant ainsi à se confronter en premier lieu à l'USADA, qu'il jugeait plus compétente. Avant de recevoir Floyd Landis le 14 mai prochain, celle-ci avait pris une mesure inédite fin mars en convoquant donc des analyses rétroactives sur des échantillons qui s'étaient révélés négatifs à l'époque, la méthode d'analyse n'étant pas aussi précise : lesquels seraient donc bien positifs à la testostérone.

Landis crie au scandale

Evidemment, celui qui est encore vainqueur du Tour de France 2006 à ce jour n'a pas tardé à réagir, criant une nouvelle fois au complot. L'Américain reproche notamment à l'USADA d'avoir empêché à l'un de ses consultants l'accès au LNDD. «Comment puis-je prouver mon innocence alors que l'USADA ne cesse de violer ses propres règles ? Je suis ulcéré par le comportement de l'USADA et du LNDD. Ensemble, ils ont transformé cette procédure en une attaque en règle contre mes droits civils et en une parodie de justice», lance-t-il notamment dans un communiqué publié sur son site internet. La balle est désormais dans le camp de l'Agence Américaine Antidopage, seule habilitée à sanctionner Floyd Landis. Convoqué le 14 mai par l'USADA, il risque une suspension de deux ans et pourrait être le deuxième vainqueur du Tour de France de l'histoire, après Maurice Garin en 1904, à être déchu de son titre, le premier pour dopage.

GDC0324

Ginnaven, Christopher C.

From: Michael Henson [m.henson@floydfairnessfund.org]
Sent: Thursday, April 26, 2007 12:02 PM
To: Suh, Maurice; Howard Jacobs; Paul Scott
Subject: Sport24 - Translated

Landis caught in the trap
4/23/07

According to l'Equipe, the retroactive analyses of seven Floyd Landis specimens clearly show the presence of exogenous testosterone. The American is facing a two-year suspension.

By Florian Egly

Crushing results

This time, Floyd Landis will not be able to hide behind a supposed exceptionally elevated natural testosterone production, nor steal away by denouncing the supposed incompetence of the laboratory at Châtenay-Malabry (LNDD). These two lines of defense put forward by the American racer since his positive test result during the Tour de France have fallen flat. According to a report in l'Equipe, seven specimens belonging to the former Yellow Jersey and preserved since the 2006 Grande Boucle have revealed the presence of exogenous testosterone, during retroactive analyses performed this last week by the LNDD. The new tests--based on the more reliable new IRMS technique (which can distinguish the level of endogenous testosterone from that due to external sources)--had been requested by the USADA, which is in charge of the Landis case. Two experts from the agency, along with two of the rider's representatives, witnessed these analyses, which thus rebuts the argument of any so-called manipulation on the part of the French laboratory.

No surprise

This is no big surprise. The revelations only confirm the American's positive test the evening of his 'fantastic' gallop to Morzine, July 20, which itself had been confirmed after analysis of that test's B sample several weeks later. Up until now, though, Landis had been able to get out of it by several maneuvers, using every recourse available, and benefiting especially from two procedural blunders by the lab at Châtenay-Malabry: a labeling error in the summary of the confirming test, and the presence of two technicians on the testing of both the A and B samples, which is prohibited by rule. Summoned by the AFLD last February, the former Phonak leader negotiated a postponement by accepting not to be at the start of the

2007 Tour de France. This allowed him to confront the USADA, which he considered more competent, first. Before meeting with Floyd Landis on next May 14, the USADA took the unprecedented step at the end of March of requiring retroactive analyses of the specimens which had tested negative at the time they were taken, the method of analysis used not being as precise.

It is these specimens that indeed seem to be positive for testosterone.

Landis cries foul

Obviously, the man who is today still the winner of the 2006 Tour de France did not waste time before reacting, once again denouncing a conspiracy. The American notably reproaches the USADA for having denied one of his consultants access to the LNDD. "How can I prove my innocence when the USADA won't stop violating its own rules? I am torn up by the behavior of the USADA and the LNDD. Together, they have transformed this process into an attack on my civil rights and a parody of justice," he asserts in a press release published on his internet site. The ball is now in the USADA's court, the only agency authorized to sanction Floyd Landis. Summoned by the USADA for May 14, he risks a suspension of two years and could be the first winner in the history of the Tour de France to be stripped of his title.

Ginnaven, Christopher C.

From: Michael Henson [michaelhenson@mac.com]
Sent: Thursday, April 26, 2007 12:13 PM
To: Suh, Maurice; Howard Jacobs; Paul Scott
Subject: L'Equipe 4/24 - French

Ressiot's follow-up with the confusing "ultra-positive" mention. Basically says that the retesting proves that Floyd used testosterone to win the Tour:

Landis archi-positif !
DAMIEN RESSIOT
867 words
24 April 2007
L'Équipe
3
French
Copyright 2007 L'Equipe "All Rights Reserved"
CYCLISME

DOPAGE

Landis archi-positif !

Les analyses rétrospectives sont claires : l'Américain était plusieurs fois positif à la testostérone sur le Tour 2006.

Il n'y a désormais plus aucun doute : Floyd Landis a bel et bien triché pour remporter le Tour de France 2006. Contrôlé positif à l'issue de

la 17 étape, l'Américain

a depuis toujours nié

s'être dopé en espérant échapper à toute sanction pour vice

de procédure. Mais les analyses rétroactives

de sept échantillons urinaires prélevés pendant la Grande Boucle ont confirmé

une prise exogène

de testostérone.

CONTRÔLÉ POSITIF à la testostérone une première fois, à l'issue de la 17 étape du dernier Tour de France, Floyd Landis, vainqueur en sursis, va devoir désormais réorienter sérieusement sa stratégie de défense. En effet, ce n'est pas une, mais plusieurs infractions aux règles antidopage que le trentenaire américain a commises en juillet 2006, puisque plusieurs de ses échantillons prélevés lors de cette même épreuve contiennent des traces de testostérone synthétique.

Après s'être opposé vigoureusement à l'idée même d'analyses complémentaires menées par le laboratoire de Châtenay-Malabry (LNDD), l'ancien leader de Phonak avait dû finalement se résoudre à cette extrémité, en vertu d'une décision prononcée par le panel (AAA) de l'Agence américaine antidopage (USADA). On comprend désormais mieux son appréhension depuis que l'on connaît le résultat des analyses supplémentaires, lesquelles ont duré une semaine en raison de la complexité de la méthode utilisée, la spectrométrie de masse (IRMS, voir ci-dessous).

Cette méthodologie, qui permet de distinguer la présence de testostérone naturelle fabriquée par l'organisme de celle produite par synthèse, livre en effet des résultats irréfutables et n'aboutit jamais à la mise en cause de faux positifs. Parmi les sept échantillons restants, analysés une nouvelle fois, plusieurs sont positifs.

Comment dès lors expliquer que des échantillons déclarés négatifs dans un premier temps puissent être déclarés positifs après de nouvelles analyses ? Tout simplement parce que, dans le cadre de la détection de routine de la testostérone, les laboratoires débutent le processus de l'analyse par l'établissement du célèbre ratio testostérone sur épitestostérone (T/E). Ce n'est que lorsque celui-ci est supérieur à 4, en vertu des règles édictées par l'Agence mondiale antidopage, que les laboratoires commencent alors une instruction plus poussée, laquelle passe, lorsqu'ils sont équipés du matériel adéquat, par l'utilisation de la méthode IRMS. Lors du Tour de France 2006, un seul des échantillons appartenant à Landis dépassait la barre fatidique des 4 (11, en l'occurrence, comme seuil de confirmation). Les sept autres échantillons prélevés sur l'Américain étaient en revanche en deçà du seuil d'instruction et n'ont pas fait l'objet de recherche par IRMS.. C'est cette nouvelle opération analytique qui a été menée à Châtenay-Malabry ces derniers jours.

Les droits

de la défense

respectés

Landis pourra-t-il, dès lors, comme il ne cesse de le faire depuis l'annonce de son premier contrôle positif, remettre en cause la crédibilité et la compétence des techniciens du LNDD, lui qui avait demandé, après s'être résigné au verdict du panel, que ces fameuses analyses rétrospectives soient effectuées au sein du laboratoire de Los Angeles (UCLA) ? Difficile.

En effet, les analyses supplémentaires opérées en France ont été menées dans le cadre de règles strictes, en aveugle (avec de nouveaux codes), avec l'adjonction d'échantillons supplémentaires (des leurres destinés à rendre toute identification aléatoire) et en présence de plusieurs experts représentant les deux parties, l'USADA et Landis (voir ci-dessous). Cette dernière précaution, imposée par l'Agence française de lutte contre le dopage (AFLD), s'est révélée extrêmement judicieuse pour préserver l'aspect contradictoire de l'expertise, et le coureur et sa cohorte d'avocats auront bien du mal à remettre en cause un processus qu'ils ont pu observer de très près. Nul doute en effet que les deux personnes présentes à Châtenay-Malabry durant les sept jours d'analyses un avocat et un scientifique auraient stoppé le processus ou crié au scandale si une irrégularité s'était produite au sein du laboratoire.

Bien entendu, ils ne devraient néanmoins pas renoncer à stigmatiser telle ou telle insuffisance, mais l'USADA, dont les deux représentants ont quitté la France dès samedi soir dernier, ont semblé totalement satisfaits par la manière dont les opérations se sont déroulées.

Désormais, et alors que cette même agence américaine s'intéresse également au financement de la campagne de défense de Landis, on attend avec curiosité la tonalité que ce dernier adoptera le 14 mai à Los Angeles, jour de sa comparution devant le panel de l'USADA.

Les débats, qui à l'heure actuelle sont évalués à une dizaine de jours, seront-ils publics, comme Landis l'avait expressément demandé ? Ce dernier, face à la nouvelle consistance des accusations, se raviserait-il et opterait-il plutôt pour une négociation intelligente ? La seule certitude, pour l'heure, c'est que l'Américain, qui s'est engagé auprès des instances françaises ad hoc (AFLD) à ne pas courir en France en 2007, pourrait également s'abstenir en 2008. Suspension oblige.

Ginnaven, Christopher C.

From: Michael Henson [m.henson@floydfairnessfund.org]
Sent: Thursday, April 26, 2007 11:58 AM
To: Suh, Maurice; Paul Scott; Howard Jacobs
Subject: Monday's L'Equipe Story - Translated

Doping: Landis overwhelmed
by Damien Ressirot
L'Equipe
4/23/07

Supplementary analyses just completed on seven Floyd Landis specimens taken during the 2006 Tour de France and originally classified as negative, have on several occasions made it possible to reveal clear traces of synthetic testosterone. The American rider, winner of the Grande Boucle but officially testing positive for a first time at the conclusion of the race (also for synthetic testosterone), must appear before an ADA panel this coming May 14 and risks a two year suspension.

After initial opposition to the retroactive analyses, Landis, confronted now with these new crushing results from the laboratory at Châtenay-Malabry (LNDD), is now going to have to readjust his defense strategy as quickly as possible. For these new positive specimens--revealed by means of the IRMS technique (which distinguishes the origin of any testosterone detected as natural or synthetic)--will support the current accusation made by the USADA (and the UCI). Moreover, Floyd Landis, who has for several months been making an effort to discredit the French laboratory, will have a great deal of trouble using that argument this time: the latest analyses, performed during this past week and completed this weekend, were carried out in the presence of two experts named by the rider and two representatives of the USADA.

Ginnaven, Christopher C.

From: Michael Henson [m.henson@floydfairnessfund.org]
Sent: Thursday, April 26, 2007 12:18 PM
To: Suh, Maurice; Howard Jacobs; Paul Scott
Subject: L'Equipe , 11/14/06 - French

The "hacking" story accusing an "associate of Landis" (later implied to be Arnie) of computer piracy at the LNDD. Note that all of these articles are written by Damien Ressiot. Ressiot is currently implicated in the Cofidis Affair for falsifying evidence and was a subject of investigation in the Vrijman Report.

Piratage à Châtenay-Malabry

DAMIEN RESSIOT

956 words

14 November 2006

L'Equipe

7

French

Copyright 2006 L'Equipe "All Rights Reserved"

TOUS SPORTS

DOPAGE

Piratage à Châtenay-Malabry

Le Laboratoire national de dépistage du dopage a été victime de plusieurs intrusions dans son système informatique.

LE MONDE DE LA LUTTE antidopage est vertigineux. Ceux qui doutent encore de la vérité de ce postulat seront attentifs au dernier thriller dans lequel se trouve plongé le Laboratoire national de dépistage du dopage de Châtenay-Malabry (LNDD), victime de pirates qui se sont introduits dans les systèmes informatiques internes.

Une plainte officielle a été déposée mardi dernier par Pierre Bordry, président de l'Agence française de lutte contre le dopage (AFLD), tutelle officielle du laboratoire depuis le 1 octobre. L'enquête a été confiée aux policiers de l'Office central de lutte contre la criminalité liée aux technologies de l'information et de la communication (OCLCTIC), qui sont entrés immédiatement en action. Car l'enjeu est de taille.

Outre le délit en lui-même, les informations contenues dans le système du laboratoire français accrédité sont en effet très sensibles et concernent des dossiers de dopage explosifs, dont les plus récents, médiatiquement parlant, sont relatifs à deux cyclistes américains, Lance Armstrong (*) et Floyd Landis, contrôlé positif à la testostérone lors du dernier Tour de France.

Qu'en pense Landis ?

Les enquêteurs de l'OCLCTIC ont donc entendu en toute hâte plusieurs membres du laboratoire, dont son directeur, le P Jacques De Ceaurriz. Leur enquête vise à identifier les auteurs de cette violation et le mobile de ce délit.

Pour ce faire, les policiers disposent d'éléments assez précis, essentiellement constitués de courriels et de courriers qui ont été distribués à différentes personnes, institutions sportives et médias.

Ces envois, tout au moins pour ceux qui concernent la France, sont constitués chacun d'une lettre de présentation datée de la première quinzaine de septembre, rédigée dans un français assez approximatif et émanant à première vue d'un Anglo-Saxon. Ces quelques lignes ont pour but d'avertir leur lecteur que les informations contenues dans les pièces jointes à cette lettre sont « très intéressantes » et se veulent révélatrices de la propension du laboratoire français à commettre des erreurs dans ses analyses.

Comment les auteurs de cette manipulation s'y sont-ils pris pour arriver, très

péniblement, à dénoncer l'incompétence supposée du LNDD ? En utilisant tout simplement quelques passages parmi les documents internes au laboratoire relatifs à des rectifications antérieures de résultats d'analyse et en les remettant en scène de manière totalement artificielle, hors de leur contexte initial. Une lecture attentive de ces pièces confirme d'ailleurs leur falsification. Les courriers ont été reconstitués. Dépourvus d'en-tête officiel du LNDD, ces documents ont dû être remis en forme par le ou les faussaires, lesquels ont rajouté des en-têtes comportant des erreurs ! Ainsi, le logo du LNDD comporte une adresse sise à « Châtenay-Malabry ».

Ces courriers ou courriels, semblables pour la plupart, ont été transmis au Comité olympique international (CIO), à l'Agence mondiale antidopage (AMA), à l'Union cycliste internationale (UCI), à Howard Jacobs, avocat américain de Floyd Landis, à différents journalistes et à certains laboratoires antidopage étrangers accrédités par l'AMA. Celui de Montréal, dirigé par le D Christiane Ayotte, a bien reçu un courriel de ce type, fin octobre, et a rapidement donné l'alerte. Jointe hier au téléphone, cette dernière nous a raconté sa stupéfaction : « J'ai effectivement reçu un e-mail signé d'un certain ³Norman Crépin², qui faisait suite à un article de presse dans lequel je démontais scientifiquement le dossier de défense constitué par Floyd Landis publié sur Internet. Le courriel me disait que le laboratoire français était le spécialiste des erreurs à répétition dans ses analyses antidopage et mettait à ma disposition, en pièces jointes, certains documents ceux dont vous venez de me parler ainsi qu'un courriel d'Olivier Rabin, le directeur scientifique de l'AMA, adressé au LNDD. La forme était suspecte, les courriers censés émaner de Châtenay-Malabry non signés par Jacques De Ceaurriz, bref, j'étais très circonspecte. J'ai avertie l'AMA et mes collègues français. Et puis, ce nom, ³Norman Crépin²... Qui est-ce ? » S'il existe bien une personne répondant à ce nom de famille salariée du laboratoire, elle n'a rien à voir avec cette affaire.

Selon nos informations, les policiers de l'OCLCTIC auraient déjà piégé l'auteur de l'envoi d'au moins un courriel, qui serait l'un des membres de l'entourage de Floyd Landis. Le nom du coureur américain revient donc de manière récurrente dans cette affaire, avec, dans son sillage, l'ombre du D Arnie Baker, entraîneur et conseiller de Landis, qui s'est chargé d'orchestrer sur Internet la défense de ce dernier axée, toujours, sur la tendance supposée du laboratoire à multiplier les erreurs.

Alors que les policiers continuent leur enquête dans cette affaire, les laboratoires antidopage continuent donc de subir pressions et attaques de tous styles. Après le dossier Olga Yegorova-EPO et le sabotage non élucidé de l'échantillon B de l'athlète russe (voir L'Équipe du 7 septembre 2001) au sein de ce même laboratoire de Châtenay-Malabry, d'autres structures accréditées par l'AMA essuient des tentatives de déstabilisation grossières et mafieuses. « Auparavant, ces stratégies étaient circonscrites à l'Amérique du Nord, soupire le D Ayotte. Désormais, les attaques personnelles à l'anglo-saxonne se multiplient. Avec l'affaire Armstrong, le dossier Landis, le LNDD est malheureusement gâté. C'est honteux. »

(*) Lance Armstrong, convaincu d'usage d'EPO lors de son premier Tour de France (1999) par le biais d'analyses rétroactives (voir L'Équipe du 23 août 2005) menées par le laboratoire français, ne cesse depuis de remettre en cause la compétence de ce dernier.

Ginnaven, Christopher C.

From: Michael Henson [michaelhenson@mac.com]
Sent: Thursday, April 26, 2007 12:24 PM
To: Suh, Maurice; Howard Jacobs; Paul Scott
Subject: L'Equipe, 7/30/06 - FRENCH

Basically a satirical column mocking Floyd in the week between the Stage 17 'A' and 'B' analysis:

SALUT LES AMISHS !
PIERRE MICHEL BONNOT
690 words
30 July 2006
L'Equipe
8
French
Copyright 2006 L'Equipe "All Rights Reserved"
SALUT LES AMISHS !

ET S'IL AVAIT VRAIMENT FAIT LE COUP ? Et si Floyd Landis avait vraiment réussi son exploit de Morzine à l'eau claire ? Ou mieux encore, par la seule grâce d'un détonant composé whisky-bière, comme il l'affirme.

Vous imaginez un peu la cruauté de la situation, le sentiment d'impuissance et d'injustice du champion ainsi privé d'un exploit définitivement corrodé par l'acide du doute. On sait, ça paraît dur à avaler. Son histoire, pas le cocktail festif invoqué. À vrai dire, s'il suffisait d'une bonne gueule de bois pour s'enfiler les Saisies et Joux-Plane sur la roue arrière, on connaît quelques joyeux drilles, dont on n'aimerait pas avoir à remonter la descente à vélo, qui seraient en train d'embouteiller l'arrivée sur Morzine.. Mais, en même temps, on a du mal à croire qu'il puisse être aussi benêt, Floyd. Le genre petit vélo équipé d'une paire de sacoches qui traînent par terre et d'un dérailleur à une seule vitesse.

Imaginez-vous un moment à l'arrivée de La Toussuire le moral dans les socquettes blanches.

Immédiatement, vous vous dites : « Tiens, et si je me collais un petit patch de testostérone, je sais bien que c'est plutôt fait pour se sculpter de beaux biscotos que pour relancer la chaudière et que je suis sûr de me faire coincer au premier contrôle mais bon, soyons fous ! »

On veut bien que le sportif professionnel américain considère généralement son corps comme un simple outil de travail totalement « customisable » et cette vieille manie européenne de lutte antidopage comme une entrave ridicule à la liberté d'exercer son job mais tout de même, ça ne tient pas.

Non, non, croyez-moi, commissaire Pillère, il y a du louche là-dessous ! Pas de doute, il a fallu qu'on lui tartine son 4-heures au beurre de cantharide ou qu'on touille son scotch avec une corne de rhinocéros pour que de mâles fluides le poussent ainsi vers la victoire.

Admettons, mais alors, qui aurait fait le coup et pourquoi ? Cherchez : un chef de bande que Landis aurait trahi, une épée à la retraite qu'un succès de l'Américain aurait trop vite poussé vers l'oubli, un taulier vexé qui aurait juré de le poursuivre en enfer et qui serait justement venu faire planer sur la Haute-Savoie une ombre plus inquiétante que celle du milan noir traquant le lapereau ? Vous voyez cette silhouette jaune glissant, subreptice, dans la chambre de Landis pour y jouer son petit remake de J'aurai ta peau avec la conviction vengeresse d'un héros de Mickey Spillane ? Vous avez trouvé le coupable idéal ? Nous aussi ! (Humour, Lance, humour...)

Maintenant, il se pourrait aussi que la dérive fatale de Floyd Landis ne soit que le fruit d'une éducation trop permissive. C'est vrai, ça, malgré leurs coiffes traditionnelles et leur phobie de la télévision, les mennonites de Farmersville, le gros bourg de Pennsylvanie où le Maillot Jaune a grandi, ne sont qu'une bande d'inquiétants

progressistes. Dissidents de la tribu anabaptiste des Anises, les mennonites se sont engagés sur la pente fatale du vice en mettant un jour les doigts dans la prise. Avec l'avènement de la fée électricité sont apparus le frigo indispensable dans le cyclisme moderne, le frigo , le bigoudi chauffant, la télé du voisin, les images du Tour de France et l'appel du vélo de course.

On sait bien qu'un jour les enfants doivent quitter le nid, ça n'est pas absolument une raison pour leur donner de l'élan en les faisant grimper sur une bicyclette.

Eût-il fait partie de la communauté originelle du « vieil ordre Amish », qui professe qu'il ne faut pas « se conformer au monde qui vous entoure » et refuse prudemment l'électricité et les pneus de tracteurs caoutchoutés, que Floyd Landis en serait encore à tenter de s'échapper de Pennsylvanie à draisienne. Et qu'il aurait plus sûrement risqué d'y laisser les semelles de ses godillots que sa réputation !



The World Anti-Doping Code

INTERNATIONAL STANDARD FOR TESTING

version 3.0

June 2003

PREAMBLE

World Anti-Doping Code *International Standard for Testing* is a mandatory *International Standard* developed as part of the World Anti-Doping Program.

The *International Standard for Testing* is extracted from the proposed ISO International Standard for Doping Control (ISO ISDC) which is being prepared by an expert group within the International Anti-Doping Arrangement (IADA) and WADA. The ISO ISDC is based on the IADA International Standard for Doping Control (ISDC)/ISO PAS 18873 (1999). WADA supports and is an active partner with IADA in developing the Proposed ISO ISDC to a full ISO standard. The ISO process is expected to be completed in mid 2004.

Version 1.0 of the *International Standard for Testing* was circulated to *Signatories* and governments for review and comments in November 2002. Version 2.0 was based on the comments and proposals received from *Signatories* and governments.

All *Signatories* and governments were consulted and have had the opportunity to review and provide comments on version 2.0. This draft version 3.0 will be presented for approval to the WADA Executive Committee on June 7th 2003.

The official text of the *International Standard for Testing* shall be maintained by WADA and shall be published in English and French. In the event of any conflict between the English and French versions, the English version shall prevail.

TABLE OF CONTENT

| | |
|---|----|
| PART ONE: INTRODUCTION, CODE PROVISIONS AND DEFINITIONS | 4 |
| 1.0 Introduction and scope | 4 |
| 2.0 Code Provisions | 5 |
| 3.0 Terms and definitions | 7 |
| 3.1 Defined terms from the <i>Code</i> | 7 |
| 3.2 Defined Terms from the <i>International Standard for Testing</i> | 10 |
| PART TWO: STANDARDS FOR TESTING | 12 |
| 4.0 Planning | 12 |
| 4.1 Objective | 12 |
| 4.2 General | 12 |
| 4.3 Requirements for establishing the <i>Registered Testing Pool</i> | 12 |
| 4.4 Requirements for collecting <i>Athlete</i> whereabouts information for the purposes of Out of Competition Testing | 13 |
| 4.5 Requirements for test distribution planning | 13 |
| 4.6 Requirements for selection of <i>Athletes</i> | 14 |
| 5.0 Notification of Athletes | 15 |
| 5.1 Objective | 15 |
| 5.2 General | 15 |
| 5.3 Requirements prior to notification of <i>Athletes</i> | 16 |
| 5.4 Requirements for notification of <i>Athletes</i> | 17 |
| 6.0 Preparing for the Sample Collection Session | 19 |
| 6.1 Objective | 19 |
| 6.2 General | 20 |
| 6.3 Requirements for preparing for the <i>Sample</i> Collection Session | 20 |
| 7.0 Conducting the Sample Collection Session | 21 |
| 7.1 Objective | 21 |
| 7.2 General | 21 |
| 7.3 Requirements prior to <i>Sample</i> collection | 21 |
| 7.4 Requirements for <i>Sample</i> collection | 22 |
| 8.0 Security/Post test administration | 23 |
| 8.1 Objective | 23 |
| 8.2 General | 24 |
| 8.3 Requirements for Security/post test administration | 24 |
| 9.0 Transport of Samples and documentation | 24 |
| 9.1 Objective | 24 |
| 9.2 General | 24 |
| 9.3 Requirements for transport of <i>Samples</i> and documentation | 25 |
| PART THREE: ANNEXES | 26 |
| Annex A - Investigating a possible failure to comply | 26 |
| Annex B - Modifications for <i>Athletes</i> with disabilities | 28 |
| Annex C - Collection of urine <i>Samples</i> | 30 |
| Annex D - Collection of blood <i>Samples</i> | 33 |
| Annex E - Urine <i>Samples</i> - Insufficient volume | 36 |
| Annex F - Urine <i>Samples</i> - Samples that do not meet laboratory pH or specific gravity guidelines | 38 |
| Annex G - Sample Collection Personnel Requirements | 40 |

PART ONE: INTRODUCTION, CODE PROVISIONS AND DEFINITIONS

1.0 Introduction and scope

The main purpose of *International Standard for Testing* is to plan for effective *Testing* and to maintain the integrity and identity of the *Samples*, from notifying the *Athlete* to transporting *Samples* for analysis.

The *International Standard for Testing* includes standards for test distribution planning, notification of *Athletes*, preparing for and conducting *Sample* collection, security/post test administration and transport of *Samples*.

The *International Standard for Testing*, including all annexes, is mandatory for all *Signatories* to the *Code*.

The World Anti-Doping Program encompasses all of the elements needed in order to ensure optimal harmonization and best practice in international and national anti-doping programs. The main elements are: the *Code* (Level 1), *International Standards* (Level 2), and Models of Best Practice (Level 3).

In the introduction to the *Code*, the purpose and implementation of the *International Standards* are summarized as follows:

"International Standards for different technical and operational areas within the anti-doping program will be developed in consultation with the Signatories and governments and approved by WADA. The purpose of the International Standards is harmonization among Anti-Doping Organizations responsible for specific technical and operational parts of the anti-doping programs. Adherence to the International Standards is mandatory for compliance with the Code. The International Standards may be revised from time to time by the WADA Executive Committee after reasonable consultation with the Signatories and governments. Unless provided otherwise in the Code, International Standards and all revisions shall become effective on the date specified in the International Standard or revision."

The standards included in the *International Standard for Testing* are extracted from the ISO International Standard for Doping Control (ISO ISDC), which also includes management and support processes for *Testing* activities

Definitions specified in the *Code* are written in italics. Additional definitions specific to the *International Standard for Testing* are underlined.

2.0 Code Provisions

The following articles in the *Code* directly address the *International Standard for Testing*:

Code Article 2 Anti-Doping Rule Violations:

2.3 Refusing, or failing without compelling justification, to submit to *Sample* collection after notification as authorized in applicable anti-doping rules or otherwise evading *Sample* collection.

2.4 Violation of applicable requirements regarding *Athlete* availability for *Out-of-Competition Testing* including failure to provide required whereabouts information and missed tests which are declared based on reasonable rules.

2.5 *Tampering*, or *Attempting* to tamper, with any part of *Doping Control*.

2.8 Administration or *Attempted* administration of a *Prohibited Substance* or *Prohibited Method* to any *Athlete*, or assisting, encouraging, aiding, abetting, covering up or any other type of complicity involving an anti-doping rule violation or any *Attempted* violation.

Code Article 3 Proof of Doping:

3.2.2 Departures from the *International Standard for Testing* which did not cause an *Adverse Analytical Finding* or other anti-doping rule violation shall not invalidate such results. If the *Athlete* establishes that departures from the *International Standard* occurred during *Testing* then the *Anti-Doping Organization* shall have the burden to establish that such departures did not cause the *Adverse Analytical Finding* or the factual basis for the anti-doping rule violation.

Code Article 5 Testing:

5.1 **Test Distribution Planning.** *Anti-Doping Organizations* conducting *Testing* shall in coordination with other *Anti-Doping Organizations* conducting *Testing* on the same *Athlete* pool:

5.1.1 Plan and implement an effective number of *In-Competition* and *Out-of-Competition* tests. Each International Federation shall establish a *Registered Testing Pool* for *International-Level Athletes* in its sport, and each *National Anti-Doping Organization* shall establish a national *Registered Testing Pool* for *Athletes* in its country. The national-level pool shall include *International-Level Athletes* from that country as well as other national-level *Athletes*. Each International Federation and *National Anti-Doping Organization* shall plan and conduct *In-Competition* and *Out-of-Competition Testing* on its *Registered Testing Pool*.

5.1.2 Make *No Advance Notice Testing* a priority.

5.1.3 Conduct *Target Testing*.

5.2 **Standards for Testing.** *Anti-Doping Organizations* conducting *Testing* shall conduct such *Testing* in conformity with the *International Standard for Testing*.

Code Article 7 Results Management:

7.3 Further Review of Adverse Analytical Finding Where Required by Prohibited List. The *Anti-Doping Organization* or other reviewing body established by such organization shall also conduct any follow-up investigation as may be required by the *Prohibited List*. Upon completion of such follow-up investigation, the *Anti-Doping Organization* shall promptly notify the *Athlete* regarding the results of the follow-up investigation and whether or not the *Anti-Doping Organization* asserts that an anti-doping rule was violated.

Code Article 10 Sanctions on Individuals:

10.10 Reinstatement Testing. As a condition to regaining eligibility at the end of a specified period of *Ineligibility*, an *Athlete* must, during any period of *Provisional Suspension* or *Ineligibility*, make him or herself available for *Out-of-Competition Testing* by any *Anti-Doping Organization* having *Testing* jurisdiction, and must, if requested, provide current and accurate whereabouts information. If an *Athlete* subject to a period of *Ineligibility* retires from sport and is removed from *Out-of-Competition Testing* pools and later seeks reinstatement, the *Athlete* shall not be eligible for reinstatement until the *Athlete* has notified relevant *Anti-Doping Organizations* and has been subject to *Out-of-Competition Testing* for a period of time equal to the period of *Ineligibility* remaining as of the date the *Athlete* had retired.

Code Article 14 Confidentiality and Reporting:

14.3 Athlete Whereabouts Information. *Athletes* who have been identified by their International Federation or *National Anti-Doping Organization* for inclusion in an *Out-of-Competition Testing* pool shall provide accurate, current location information. The International Federations and *National Anti-Doping Organizations* shall coordinate the identification of *Athletes* and the collecting of current location information and shall submit it to *WADA*.

WADA shall make this information accessible to other *Anti-Doping Organizations* having authority to test the *Athlete* as provided in Article 15. This information shall be maintained in strict confidence at all times; shall be used exclusively for purposes of planning, coordinating or conducting *Testing*; and shall be destroyed after it is no longer relevant for these purposes.

14.5 Doping Control Information Clearing House. *WADA* shall act as a central clearing house for *Doping Control Testing* data and results for *International-Level Athletes* and national-level *Athletes* that have been included in their *National Anti-Doping Organization's Registered Testing Pool*. To facilitate coordinated test distribution planning and to avoid unnecessary duplication in *Testing* by the various *Anti-Doping Organizations*, each *Anti-Doping Organization* shall report all *In-Competition* and *Out-of-Competition* tests on such *Athletes* to the *WADA* clearinghouse as soon as possible after such tests have been conducted. *WADA* shall make this information accessible to the *Athlete*, the *Athlete's* National Federation, *National Olympic Committee* or *National Paralympic Committee*, *National Anti-Doping Organization*, International Federation, and the International Olympic Committee or International Paralympic Committee. Private information regarding an *Athlete* shall be maintained by *WADA* in strict confidence. *WADA* shall, at least annually, publish statistical reports summarizing such information.

Code Article 15 Clarification of Doping Control Responsibilities:

15.1 Event Testing. The collection of *Samples* for *Doping Control* does and should take place at both *International Events* and *National Events*. However, only a single organization should be responsible for initiating and directing *Testing* during an *Event*. At *International Events*, the collection of *Doping Control Samples* shall be initiated and directed by the

international organization which is the ruling body for the *Event* (e.g., the IOC for the Olympic Games, the International Federation for a World Championship, and PASO for the Pan American Games). If the international organization decides not to conduct any *Testing* at such an *Event*, the *National Anti-Doping Organization* for the country where the *Event* occurs may, in coordination with and with the approval of the international organization or *WADA*, initiate and conduct such *Testing*. At *National Events*, the collection of *Doping Control Samples* shall be initiated and directed by the designated *National Anti-Doping Organization* of that country.

15.2 Out-of-Competition Testing. *Out-of-Competition Testing* is and should be initiated and directed by both international and national organizations. *Out-of-Competition Testing* may be initiated and directed by: (a) *WADA*; (b) the IOC or IPC in connection with the Olympic Games or Paralympic Games; (c) the *Athlete's* International Federation; (d) the *Athlete's National Anti-Doping Organization*; or (e) the *National Anti-Doping Organization* of any country where the *Athlete* is present. *Out-of-Competition Testing* should be coordinated through *WADA* in order to maximize the effectiveness of the combined *Testing* effort and to avoid unnecessary repetitive *Testing* of individual *Athletes*.

15.4 Mutual Recognition. Subject to the right to appeal provided in Article 13, the *Testing*, therapeutic use exemptions and hearing results or other final adjudications of any *Signatory* which are consistent with the *Code* and are within that *Signatory's* authority, shall be recognized and respected by all other *Signatories*. *Signatories* may recognize the same actions of other bodies which have not accepted the *Code* if the rules of those bodies are otherwise consistent with the *Code*.

3.0 Terms and definitions

3.1 Defined terms from the Code

Adverse Analytical Finding: A report from a laboratory or other approved *Testing* entity that identifies in a *Specimen* the presence of a *Prohibited Substance* or its *Metabolites* or *Markers* (including elevated quantities of endogenous substances) or evidence of the *Use* of a *Prohibited Method*.

Anti-Doping Organization: A *Signatory* that is responsible for adopting rules, for initiating, implementing or enforcing any part of the *Doping Control* process. This includes, for example, the International Olympic Committee, the International Paralympic Committee, other *Major Event Organizations* that conduct *Testing* at their *Events*, *WADA*, International Federations, and *National Anti-Doping Organizations*.

Athlete: For purposes of *Doping Control*, any *Person* who participates in sport at the international level (as defined by each International Federation) or national level (as defined by each *National Anti-Doping Organization*) and any additional *Person* who participates in sport at a lower level if designated by the *Person's National Anti-Doping Organization*. For purposes of anti-doping information and education, any *Person* who participates in sport under the authority of any *Signatory*, government, or other sports organization accepting the *Code*.

Code: The World Anti-Doping Code.

Competition: A single race, match, game or singular athletic contest. For example, the finals of the Olympic 100-meter dash. For stage races and other athletic contests where prizes are awarded on a daily or other interim basis, the distinction between a *Competition* and an *Event* will be as provided in the rules of the applicable International Federation.

Consequences of Anti-Doping Rules Violations: An *Athlete's* or other *Person's* violation of an anti-doping rule may result in one or more of the following: (a) Disqualification means the *Athlete's* results in a particular *Competition* or *Event* are invalidated, with all resulting consequences including forfeiture of any medals, points and prizes; (b) Ineligibility means the *Athlete* or other *Person* is barred for a specified period of time from participating in any *Competition* or other activity or funding as provided in Article 10.9; and (c) Provisional Suspension means the *Athlete* or other *Person* is barred temporarily from participating in any *Competition* prior to the final decision at a hearing conducted under Article 8 (Right to a Fair Hearing).

Doping Control: The process including test distribution planning, *Sample* collection and handling, laboratory analysis, results management, hearings and appeals.

Event: A series of individual *Competitions* conducted together under one ruling body (e.g., the Olympic Games, FINA World Championships, or Pan American Games).

In-Competition: For purposes of differentiating between *In-Competition* and *Out-of-Competition Testing*, unless provided otherwise in the rules of an International Federation or other relevant *Anti-Doping Organization*, an *In-Competition* test is a test where an *Athlete* is selected for *Testing* in connection with a specific *Competition*.

Independent Observer Program: A team of observers, under the supervision of WADA, who observe the *Doping Control* process at certain *Events* and report on observations. If WADA is *Testing In-Competition* at an *Event*, the observers shall be supervised by an independent organization.

Ineligibility: See *Consequences of Anti-Doping Rules Violations* above.

International Event: An *Event* where the International Olympic Committee, the International Paralympic Committee, an International Federation, a *Major Event Organization*, or another international sport organization is the ruling body for the *Event* or appoints the technical officials for the *Event*.

International-Level Athlete: *Athletes* designated by one or more International Federations as being within the *Registered Testing Pool* for an International Federation.

International Standard: A standard adopted by WADA in support of the *Code*. Compliance with an *International Standard* (as opposed to another alternative standard, practice or procedure) shall be sufficient to conclude that the procedures addressed by the *International Standard* were performed properly.

Minor: A natural *Person* who has not reached the age of majority as established by the applicable laws of his or her country of residence.

National Anti-Doping Organization: The entity(ies) designated by each country as possessing the primary authority and responsibility to adopt and implement anti-doping rules, direct the collection of *Samples*, the management of test results, and the conduct of hearings, all at the national level. If this designation has not been made by the competent public authority (ies), the entity shall be the country's *National Olympic Committee* or its designee.

National Olympic Committee: The organization recognized by the International Olympic Committee. The term *National Olympic Committee* shall also include the National Sport Confederation in those countries where the National Sport Confederation assumes typical *National Olympic Committee* responsibilities in the anti-doping area.

No Advance Notice: A *Doping Control* which takes place with no advance warning to the *Athlete* and where the *Athlete* is continuously chaperoned from the moment of notification through *Sample* provision.

Out-of-Competition: Any *Doping Control* which is not *In-Competition*.

Prohibited List: The List identifying the *Prohibited Substances* and *Prohibited Methods*.

Provisional Suspension: See *Consequences* above.

Registered Testing Pool: The pool of top level *Athletes* established separately by each International Federation and *National Anti-Doping Organization* who are subject to both *In-Competition* and *Out-of-Competition Testing* as part of that International Federation's or Organization's test distribution plan.

Sample/Specimen: Any biological material collected for the purposes of *Doping Control*.

Signatories: Those entities signing the *Code* and agreeing to comply with the *Code*, including the International Olympic Committee, International Federations, International Paralympic Committee, *National*

Olympic Committees, National Paralympic Committees, Major Event Organizations, National Anti-Doping Organizations, and WADA.

Target Testing: Selection of *Athletes* for *Testing* where specific *Athletes* or groups of *Athletes* are selected on a non-random basis for *Testing* at a specified time.

Testing: The parts of the *Doping Control* process involving test distribution planning, *Sample* collection, *Sample* handling, and *Sample* transport to the laboratory.

WADA: The World Anti-Doping Agency.

3.2 Defined Terms from the *International Standard for Testing*

Blood Collection Official: An official who is qualified to and has been authorized by the *ADO* to collect a blood *Sample* from an *Athlete*.

Chain of Custody: The sequence of individuals or organizations who have the responsibility for a *Sample/specimen* from the provision of the sample/specimen until the *Sample/specimen* has been received for analysis.

Chaperone: An official who is trained and authorized by the *ADO* to carry out specific duties including notification of the *Athlete* selected for *Sample* collection, accompanying and observing the *Athlete* until arrival at the *Doping Control Station*, and/or witnessing and verifying the provision of the *Sample* where the training qualifies him/her to do so.

Doping Control Officer: An official who has been trained and authorised by the *ADO* with delegated responsibility for the on-site management of a *Sample Collection Session*.

Doping Control Station: The location where the *Sample Collection Session* will be conducted.

Failure to Comply: A term used to describe *Anti-Doping Rule Violations* in Articles 2.3, 2.4, 2.5 and 2.8 of the Code.

Sample Collection Equipment: Containers or apparatus used to directly collect or hold the *Athlete's Specimen* at any time during the *Sample* collection process. *Sample Collection Equipment* shall, as a minimum, consist of:

- For urine *Sample* collection:
 - Collection vessels for collecting the urine *Sample* as it leaves the *Athlete's* body;
 - Sealable and tamper-evident bottles and lids for securing the urine *Sample*;

- For blood *Sample* collection:
 - Needles for collecting the blood *Sample*;
 - Blood tubes with sealable and tamper-evident devices for holding the blood *Sample*.

Sample Collection Personnel: A collective term for qualified officials authorised by the *ADO* who may carry out or assist with duties during the *Sample Collection Session*.

Sample Collection Session: All of the sequential activities that directly involve the *Athlete* from notification until the *Athlete* leaves the Doping Control Station after having provided his/her *Sample/s*.

Weighted: A ranking method of selecting *Athletes* using criteria where the ranking is based on the potential risk of doping and possible doping patterns.

PART TWO: STANDARDS FOR TESTING

4.0 Planning

4.1 Objective

The objective is to plan and implement an effective distribution of *Athlete* tests.

4.2 General

Planning starts with establishing criteria for *Athletes* to be included in a *Registered Testing Pool* and ends with selecting *Athletes* for *Sample* collection.

The main activities are information gathering, risk evaluation, and developing, monitoring, evaluating and modifying the test distribution plan.

4.3 Requirements for establishing the *Registered Testing Pool*

4.3.1 The *Anti-Doping Organization* (ADO) shall define and document the criteria for *Athletes* to be included in a *Registered Testing Pool*. This shall include as a minimum:

- For International Federations (IFs):
Athletes who compete at a high level of international competition, and
- For *National Anti-Doping Organizations*:
Athletes who are part of national teams in Olympic and Paralympic sports and recognised national federations.

The criteria shall be reviewed at least annually and updated if required.

4.3.2 The ADO shall include *Athletes* under their authority in the *Registered Testing Pool* who are serving periods of *Ineligibility* or *Provisional Suspensions* as *Consequences of Anti-Doping Rules Violations*.

4.3.3 The *Registered Testing Pool* shall be reviewed and updated regularly to reflect changes in *Athletes'* competing levels to ensure additions to or removals from the pool as required.

4.4 Requirements for collecting *Athlete* whereabouts information for the purposes of Out of Competition Testing

4.4.1 The ADO shall define procedures and/or systems for:

- a) Collecting, maintaining and monitoring sufficient whereabouts information to ensure that *Sample* collection can be planned and conducted at *No Advance Notice* for all *Athletes* included in the *Registered Testing Pool*, and
- b) When *Athletes* fail to provide accurate and timely whereabouts information, taking appropriate action to ensure the information stays up to date and complete.

4.4.2 As a minimum the following *Athlete* whereabouts information shall be collected:

- a) Name
- b) Sport/discipline,
- c) Home address
- d) Contact phone numbers
- e) Training times and venues
- f) Training camps
- g) Travel plans
- h) Competition schedule
- i) Disability if applicable, including the requirement for third party involvement in notification.

4.5 Requirements for test distribution planning

4.5.1 The ADO shall, as a minimum, evaluate the potential risk of doping and possible doping pattern for each sport and/or discipline based on:

- a) Physical demands of the sport and possible performance enhancing effect that doping may elicit;
- b) Available doping analysis statistics;
- c) Available research on doping trends;
- d) Training periods and *Competition* season.

4.5.2 The ADO shall develop and document a test distribution plan based on information determined in 4.5.1, the number of *Athletes* per sport/discipline in the *Registered Testing Pool* and the evaluation outcomes of previous test distribution planning cycles.

4.5.3 The ADO shall allocate the number of *Sample* collections by type of *Sample* collection for each sport/discipline, including *No Advance Notice*, *Out-of-Competition*, *In-Competition*, blood and urine *Sample* collection, as required to achieve effective deterrence.

4.5.4 The ADO shall establish a system whereby the test distribution plan is reviewed and, if necessary, updated on a regular basis in order to incorporate new information and take into account *Sample* collection from *Athletes* in the *Registered Testing Pool* by other ADOs.

4.5.5 The ADO shall establish a system for maintaining test distribution planning data. Such data shall be used to assist with determining whether modifications to the plan are necessary. This information shall include as a minimum:

For each test:

- a) The sport/discipline;
- b) The country represented by the *Athlete* (if applicable);
- c) The type of *Sample* collection (*No Advance Notice*, *Out-of-Competition*, *In-Competition* or advance notice);
- d) The date of *Sample* collection; and
- e) The country in which the *Sample* collection occurred.

In addition, for each *Adverse Analytical Finding*:

- a) Dates of *Sample* collection and analysis;
- b) Class of substance/s found;
- c) Actual substance/s detected;
- d) *Sanctions of Anti-Doping Rules Violations*, if any.

4.5.6 The ADO shall ensure that the athlete support personnel shall not be involved in the test distribution planning for their athletes.

4.5.7 In planning and conducting tests at *International Event*, and where the relevant IF does not have a doping control program that complies with this standard, the *National Anti-Doping Organization* shall be the preferred *Sample* collection supplier.

4.6 Requirements for selection of *Athletes*

4.6.1 In accordance with the number of *Sample* collections allocated to each sport/discipline in the test distribution plan, the ADO shall select *Athletes* for *Sample* collection using *Target Testing*, Weighted and random selection methods.

4.6.2 As a minimum, the *ADO* shall consider *Target Testing Athletes* based on the following information:

- a) Injury;
- b) Withdrawal or absence from expected *Competition*;
- c) Going into or coming out of retirement;
- d) Behaviour indicating doping;
- e) Sudden major improvements in performance;
- f) Changes in *Athlete* whereabouts information that can indicate a potential increase in the risk of doping, including moving to a remote location;
- g) *Athlete* sport performance history;
- h) Details of past *Doping Controls*;
- i) *Athlete* reinstatement after a period of *Ineligibility*; and
- j) Reliable information from a third party.

4.6.3 An *ADO* may select *Athletes* under their authority for *Sample* collection who are not included in the *Registered Testing Pool* defined in 4.3.1 and 4.3.2.

4.6.4 Where the *ADO* authorises a Doping Control Officer (DCO) to select *Athletes* for *Sample* collection, the *ADO* shall provide selection criteria to the DCO in accordance with the test distribution plan.

4.6.5 Following the selection of an *Athlete* for *Sample* collection and prior to notification of the *Athlete*, the *ADO* and/or DCO shall ensure *Athlete* selection decisions are disclosed only to those who need to know in order to ensure the *Athlete* can be notified and tested on a *No Advance Notice* basis.

5.0 Notification of Athletes

5.1 Objective

To ensure that the selected *Athlete* is notified, the rights of the *Athlete* are maintained, there are no opportunities to manipulate the *Sample* to be provided and the notification is documented.

5.2 General

Notification of *Athletes* starts when the *ADO* initiates the notification of the selected *Athlete* and ends when the *Athlete* arrives at the Doping Control Station or when the *Athlete's* possible failure to comply is brought to the *ADO's* attention.

The main activities are:

- a) Appointment of DCOs, Chaperones and other Sample Collection Personnel;
- b) Locating the *Athlete* and confirming his/her identity;
- c) Informing the *Athlete* that he/she has been selected to provide a *Sample* and of his/her rights and responsibilities;
- d) For *No Advance Notice Sample* collection, continuously chaperoning the *Athlete* from the time of notification to the arrival at the designated Doping Control Station; and
- e) Documenting the notification.

5.3 Requirements prior to notification of *Athletes*

5.3.1 *No Advance Notice* shall be the notification method for *Out-of-Competition Sample* collection whenever possible.

5.3.2 To conduct or assist with Sample Collection Sessions, the ADO shall appoint and authorise Sample Collection Personnel who have been trained for their assigned responsibilities, who do not have a conflict of interest in the outcome of the *Sample* collection, and who are not *Minors*.

5.3.3 Sample Collection Personnel shall have official identification that is provided and controlled by the ADO. The minimum identification requirement is an official card/document naming the ADO through which they have been authorised. For DCOs, additional identification requirements shall include their name, their photograph and the card's/document's expiry date. For Blood Collection Officials additional identification requirements include evidence of their professional training in the collection of blood *Samples*.

5.3.4 The ADO shall establish criteria to validate the identity of an *Athlete* selected to provide a *Sample*. This ensures the selected *Athlete* is the *Athlete* who is notified.

5.3.5 The ADO, DCO or Chaperone, as applicable, shall establish the location of the selected *Athlete* and plan the approach and timing of notification, taking into consideration the specific circumstances of the sport/*Competition* and the situation in question.

5.3.6 For *Out-of-Competition Sample* collection, the ADO shall establish criteria to ensure that reasonable attempts are made to notify *Athletes* of their selection for *Sample* collection.

5.3.7 Reasonable attempts shall be defined by the ADO and at a minimum shall consider alternative times of day/evening and alternative locations over a specified period of time from the initial notification attempt.

5.3.8 The *ADO* shall establish a system for logging *Athlete* notification attempt/s and outcome/s.

5.3.9 The *Athlete* shall be the first one notified that he/she has been selected for *Sample* collection except where prior contact with a third party is required as specified in 5.3.10.

5.3.10 The *ADO/DCO/Chaperone*, as applicable, shall consider whether a third party is required to be notified prior to notification of the *Athlete* when the *Athlete* is a *Minor*, where required by an *Athlete's* disability as provided for in Annex B - Modifications for *Athletes* with disabilities, or in situations where an interpreter is required for the notification.

5.3.11 If the *Athlete* can not be contacted after having made reasonable attempts using the information supplied in 4.4.2 and logging the attempts in accordance with 5.3.8, the *DCO* or *ADO*, as applicable, shall institute Annex A – Investigating a possible failure to comply.

5.3.12 The *ADO* shall not re-schedule or change a *Sample* collection from *No Advance Notice* to advance notice except where an unexpected situation forces the need for an advanced notice *Sample* collection. Any such decision shall be recorded.

5.3.13 Notification for advance notice *Sample* collection shall be by any means that indicates the *Athlete* received the notice.

5.4 Requirements for notification of *Athletes*

5.4.1 When initial contact is made, the *ADO*, *DCO* or *Chaperone*, as applicable, shall ensure that the *Athlete* and/or a third party if required in accordance with 5.3.10, is informed:

- a) That the *Athlete* is required to undergo a *Sample* collection;
- b) Of the authority under which the *Sample* collection is to be conducted;
- c) Of the type of *Sample* collection and any conditions that need to be adhered to prior to the *Sample* collection;
- d) Of the *Athlete's* rights, including the right to:
 - i. Have a representative and, if required, an interpreter;
 - ii. Ask for additional information about the *Sample* collection process;
 - iii. Request a delay in reporting to the Doping Control Station for valid reasons; and
 - iv. Request modifications as provided for in Annex B – Modifications for *Athletes* with disabilities.
- e) Of the *Athlete's* responsibilities, including the requirement to:

- i. Remain within sight of the DCO/Chaperone at all times from the first moment of in-person notification by the DCO/Chaperone until the completion of the *Sample* collection procedure;
 - ii. Produce identification in accordance with 5.3.4; and
 - iii. Comply with *Sample* collection procedures and the possible consequences of failure to comply; and
 - iv. Report to the Doping Control Station, unless delayed for valid reasons, as soon as possible and within 60 minutes of notification for a *No Advance Notice Sample* collection and 24 hours of receipt of notification for an advance notice *Sample* collection.
- f) Of the location of the Doping Control Station.

5.4.2 When in-person contact is made, the DCO/Chaperone shall:

- a) From this time until the *Athlete* leaves the Doping Control Station at the end of his/her Sample Collection Session, keep the *Athlete* under observation at all times.
- b) Identify themselves to the *Athlete* using their official ADO identification card/document;
- c) Confirm the *Athlete's* identity as per the criteria established in 5.3.4. Any failure to confirm the identity of the *Athlete* shall be documented. In such cases, the DCO responsible for conducting the Sample Collection Session shall decide whether it is appropriate to report the situation in accordance with Annex A – Investigating a possible failure to comply.

5.4.3 The Chaperone/DCO shall then have the *Athlete* sign an appropriate form to acknowledge and accept the notification. If the *Athlete* refuses to sign that he/she has been notified or evades the notification, the Chaperone/DCO shall inform the *Athlete* of the consequences of failing to comply if possible, and the Chaperone (if not the DCO) shall immediately report all relevant facts to the DCO. When possible the DCO shall continue to collect a *Sample*. The DCO shall document the facts and report the circumstances to the ADO. The DCO and ADO shall follow the steps prescribed in Annex A – Investigating a possible failure to comply.

5.4.4 The DCO/Chaperone shall consider any reasonable request by the *Athlete* to delay reporting to the Doping Control Station within 60 mins of acknowledgement and acceptance of notification and approve or reject such requests as appropriate in accordance with 5.4.5 and 5.4.6. The DCO shall document the reasons for any such delay that may require further investigation by the ADO. The first urine *Sample* post notification shall be collected.

5.4.5 A DCO may accept a request from an *Athlete* to delay reporting to the Doping Control Station beyond 60 mins, and/or once the athlete arrives at the Doping Control Station and wishes to leave if the *Athlete*

can be continuously chaperoned during the delay and if the request relates to the following activities:

- a) Participation in a victory ceremony;
- b) Fulfilment of media commitments;
- c) Competing in further *competitions*;
- d) Performing a warm down;
- e) Obtaining necessary medical treatment;
- f) Locating a representative and/or interpreter.

The DCO shall document the reasons for delay in reporting to the Doping Control Station and/or reasons for leaving the Doping Control Station once arriving that may require further investigation by the *ADO*.

5.4.6 A DCO/Chaperone shall reject a request for delay from an *Athlete* if it will not be possible for the *Athlete* to be continuously chaperoned.

5.4.7 When an *Athlete* notified of an advance notice *Sample* collection does not report to the Doping Control Station at the designated time, the DCO shall use his/her judgement whether to attempt to contact the *Athlete*. At a minimum, the DCO shall wait 30 minutes after the appointed time before departing. If the *Athlete* still has not reported by the time the DCO departs, the DCO shall follow the requirements of Annex A – Investigating a possible failure to comply.

5.4.8 If the *Athlete* reports to the Doping Control Station after the minimum waiting time and prior to the DCO's departure, the DCO shall decide as to whether to process a possible failure to comply. If at all possible the DCO shall proceed with collecting a *Sample*, and shall document the details of the delay in the *Athlete* reporting to the Doping Control Station.

5.4.9 If, while keeping the *Athlete* under observation, Sample Collection Personnel observe any matter with potential to compromise the test, the circumstances shall be reported to and documented by the DCO. If deemed appropriate by the DCO, the DCO shall follow the requirements of Annex A – Investigating a possible failure to comply.

6.0 Preparing for the Sample Collection Session

6.1 Objective

To prepare for the Sample Collection Session in a manner that ensures that the session can be conducted efficiently and effectively.

6.2 General

Preparing for the Sample Collection Session starts with the establishment of a system for obtaining relevant information for effective conduct of the session and ends when it is confirmed that the Sample Collection Equipment conforms to the specified criteria.

The main activities are:

- a) Establishing a system for collecting details regarding the Sample Collection Session;
- b) Establishing criteria for who may be authorised to be present during a Sample Collection Session;
- c) Ensuring that the Doping Control Station meets the minimum criteria prescribed in 6.3.2;
- d) Ensuring that Sample Collection Equipment used by the ADO meets the minimum criteria prescribed in 6.3.4.

6.3 Requirements for preparing for the Sample Collection Session

6.3.1 The ADO shall establish a system for obtaining all the information necessary to ensure that the Sample Collection Session can be conducted effectively, including special requirements to meet the needs of *Athletes* with disabilities as provided in Annex B – Modifications for *Athletes* with disabilities.

6.3.2 The DCO shall use a Doping Control Station which, at a minimum, ensures the *Athlete's* privacy and is used solely as a Doping Control Station for the duration of the Sample Collection Session. The DCO shall record any significant deviations from these criteria.

6.3.3 The ADO shall establish criteria for who may be authorised to be present during the Sample Collection Session in addition to the Sample Collection Personnel. At a minimum the criteria shall include:

- a) An *Athlete's* entitlement to be accompanied by a representative and/or interpreter during the Sample Collection Session except when the *Athlete* is passing a urine *Sample*.
- b) A *Minor Athlete's* entitlement, and the witnessing DCO/Chaperone's entitlement to have a representative observe the Chaperone when the *Minor Athlete* is passing a urine *Sample*, but without the representative directly observing the passing of the *Sample* unless requested to do so by the *Minor Athlete*.
- c) An *Athlete* with a disability's entitlement to be accompanied by a representative as provided for in Annex B - Modifications for *Athletes* with disabilities.

- d) A WADA Independent Observer where applicable under the *Independent Observer Program*. The WADA Independent Observer shall not directly observe the passing of a urine *Sample*.

6.3.4 The DCO shall only use Sample Collection Equipment systems that are authorised by the ADO, which at a minimum, shall meet the following criteria. They shall:

- a) Have a unique numbering system incorporated into all bottles, containers, tubes or any other item used to seal the *Athlete's Sample*;
- b) Have a sealing system that is tamper evident;
- c) Ensure the identity of the *Athlete* is not evident from the equipment itself;
- d) Ensure that all equipment is clean and sealed prior to use by the *Athlete*.

7.0 Conducting the Sample Collection Session

7.1 Objective

To conduct the Sample Collection Session in a manner that ensures the integrity, security and identity of the *Sample* and respects the privacy of the *Athlete*.

7.2 General

The Sample Collection Session starts with defining overall responsibility for the conduct of the Sample Collection Session and ends once the *Sample* collection documentation is complete.

The main activities are:

- a) Preparing for collecting the *Sample*;
- b) Collecting the *Sample*; and
- c) Documenting the *Sample* collection.

7.3 Requirements prior to Sample collection

7.3.1 The ADO shall be responsible for the overall conduct of the Sample Collection Session with specific responsibilities delegated to the DCO.

7.3.2 The DCO shall ensure that the *Athlete* is informed of his/her rights and responsibilities as specified in 5.4.1.

7.3.3 The DCO shall provide the *Athlete* with the opportunity to hydrate.

7.3.4 The *Athlete* shall only leave the Doping Control Station under continuous observation by the DCO/Chaperone and with the approval of the DCO. The DCO shall consider any reasonable request by the *Athlete* to leave the Doping Control Station, as specified in 5.4.5 and 5.4.6, until the *Athlete* is able to provide a *Sample*.

7.3.5 If the DCO gives approval for the *Athlete* to leave the Doping Control Station, the DCO shall agree with the *Athlete* on:

- a) The purpose of the *Athlete* leaving the Doping Control Station; and
- b) The time of return (or return upon completion of an agreed activity).

The DCO shall document this information and the actual time of the *Athlete's* departure and return.

7.4 Requirements for *Sample* collection

7.4.1 The DCO shall collect the *Sample* from the *Athlete* according to the following protocol/s for the specific type of *Sample* collection:

- a) Annex C: Collection of urine *Samples*
- b) Annex D: Collection of blood *Samples*

7.4.2 Any behaviour by the *Athlete* and/or persons associated with the *Athlete* or anomalies with potential to compromise the *Sample* collection shall be recorded. If appropriate, the *ADO* and/or DCO, as applicable, shall institute Annex A – Investigating a possible failure to comply.

7.4.3 If there are doubts as to the origin or authenticity of the *Sample*, the *Athlete* shall be asked to provide an additional *Sample*. If the *Athlete* refuses to provide an additional *Sample* the DCO shall institute Annex A – Investigating a possible failure to comply.

7.4.4 The DCO shall provide the *Athlete* with the opportunity to document any concerns he/she may have about how the session was conducted.

7.4.5 In conducting the Sample Collection Session the following information shall be recorded as a minimum:

- a) Date, time and type of notification (*No Advance Notice*, advance notice, *In-Competition* or *Out-of-Competition*);
- b) Date and time of *Sample* provision;
- c) The name of the *Athlete*;
- d) The date of birth of the *Athlete*;

- e) The gender of the *Athlete*;
- f) The *Athlete's* home address and telephone number;
- g) The *Athlete's* sport and discipline;
- h) The *Sample* code number;
- i) The name and signature of the Chaperone who witnessed the urine *Sample* provision;
- j) The name and signature of the Blood Collection Official who collected the blood *Sample*, where applicable;
- k) Required laboratory information on the *Sample*;
- l) Medications and supplements taken and recent blood transfusion details if applicable, within the timeframe specified by the lab as declared by the *Athlete*;
- m) Any irregularities in procedures;
- n) *Athlete* comments or concerns regarding the conduct of the session, if provided;
- o) The name and signature of the *Athlete*;
- p) The name and signature of the *Athlete's* representative, if required; and
- q) The name and signature of the DCO.

7.4.6 The *Athlete* and DCO shall sign appropriate documentation to indicate their satisfaction that the documentation accurately reflects the details of the *Athlete's Sample Collection Session*, including any concerns recorded by the *Athlete*. The *Athlete's* representative shall sign on behalf of the *Athlete* if the *Athlete* is a *Minor*. Other persons present who had a formal role during the *Athlete's Sample Collection Session* may sign the documentation as a witness of the proceedings.

7.4.7 The DCO shall provide the *Athlete* with a copy of the records of the Sample Collection Session that have been signed by the *Athlete*.

8.0 Security/Post test administration

8.1 Objective

To ensure that all *Samples* collected at the Doping Control Station and *Sample* collection documentation are securely stored prior to their departure from the Doping Control Station.

8.2 General

Post test administration begins when the *Athlete* has left the Doping Control Station after providing his/her *Sample/s*, and ends with preparation of all of the collected *Samples* and documentation for transport.

8.3 Requirements for Security/post test administration

8.3.1 The *ADO* shall define criteria ensuring that any sealed *Sample* will be stored in a manner that protects its integrity, identity and security prior to transport from the Doping Control Station. The *DCO* shall ensure that any sealed *Sample* is stored in accordance with these criteria.

8.3.2 Without exception, all *Samples* collected shall be sent for analysis to a *WADA* accredited laboratory or as otherwise approved by *WADA*.

8.3.3 The *ADO/DCO* shall develop a system to ensure that the documentation for each sealed *Sample* is completed and securely handled.

8.3.4 The *ADO* shall develop a system to ensure that, where required, instructions for the type of analysis to be conducted are provided to the *WADA* accredited laboratory or as otherwise approved by *WADA*.

9.0 Transport of Samples and documentation

9.1 Objective

- a) To ensure that *Samples* and related documentation arrive at the *WADA* accredited laboratory or as otherwise approved by *WADA* in proper condition to do the necessary analysis, and
- b) To ensure the *Sample Collection Session* documentation is sent by the *DCO* to the *ADO* in a secure and timely manner.

9.2 General

Transport starts when the sealed *Samples* and documentation leave the Doping Control Station and ends with the confirmed receipt of the *Samples* and *Sample* collection documentation at their intended destinations.

The main activities are arranging for the secure transport of *Samples* and related documentation to the *WADA* accredited laboratory or as otherwise approved by *WADA*, and arranging for the secure transport of *Sample* collection documentation to the *ADO*.

9.3 Requirements for transport of *Samples* and documentation

9.3.1 The *ADO* shall authorise a transport system that ensures *Samples* and documentation will be transported in a manner that protects their integrity, identity and security.

9.3.2 The *ADO* shall develop a system for recording the Chain of Custody of the *Samples* and *Sample* collection documentation which includes confirming that both the *Samples* and *Sample* collection documentation have arrived at their intended destinations.

9.3.3 Sealed *Samples* shall always be transported to the *WADA* accredited laboratory or as otherwise approved by *WADA*, using the *ADO's* authorised transport method as soon as practicable after the completion of the Sample Collection Session.

9.3.4 Documentation identifying the *Athlete* shall not be included with the *Samples* or documentation sent to the *WADA* accredited laboratory or as otherwise approved by *WADA*.

9.3.5 The DCO shall send all relevant Sample Collection Session documentation to the *ADO* using the *ADO's* authorised transport method as soon as practicable after the completion of the Sample Collection Session.

9.3.6 Chain of Custody shall be checked by the *ADO* if receipt of either the *Samples* with accompanying documentation or *Sample* collection documentation is not confirmed at their intended destination or a *Sample's* integrity or identity may have been compromised during transport. In this instance, the *ADO* shall consider whether the *Sample* should be voided.

PART THREE: ANNEXES

Annex A - Investigating a possible failure to comply

A.1 Objective

To ensure that any matters occurring before, during or after a Sample Collection Session that may lead to a determination of a failure to comply are assessed, acted upon and documented.

A.2 Scope

Investigating a possible failure to comply begins when the *ADO* or a *DCO* becomes aware of a matter with the potential to compromise an *Athlete's* test and ends when the *ADO* takes appropriate follow-up action based on the outcomes of its investigation into the possible failure to comply.

A.3 Responsibility

A.3.1 The *ADO* is responsible for ensuring that:

- a) Any matters with the potential to compromise an Athlete's test are assessed to determine if a possible failure to comply has occurred;
- b) All relevant information, including information from the immediate surroundings when applicable, is obtained as soon as possible or when practicable to ensure that all knowledge of the matter can be reported and be presented as possible evidence; and
- c) Appropriate documentation is completed to report any possible failure to comply.

A.3.2 Sample Collection Personnel are responsible for reporting to the *DCO* any matter with the potential to compromise a test, and the *DCO* is responsible for reporting such matters to the *ADO*.

A.4 Requirements

A.4.1 Any matters with the potential to compromise the test shall be reported as soon as practicable.

A.4.2 If the matter has potential to compromise the test, the *Athlete* shall be notified if possible:

- a) Of the possible consequences;
- b) That a possible failure to comply will be investigated by the *ADO* and appropriate follow-up action will be taken.

A.4.3 The necessary information about the possible failure to comply shall be obtained from all relevant sources as soon as possible and recorded.

A.4.4 If possible, the *Athlete's Sample Collection Session* shall be completed.

A.4.5 The *ADO* shall establish a system for ensuring that the outcomes of its investigation into the possible failure to comply are considered for results management action and, if applicable, for further planning and *Testing*.

Annex B - Modifications for *Athletes* with disabilities

B.1 Objective

To ensure that the special needs of *Athletes* with disabilities are provided as much as possible in relation to the provision of a *Sample*.

B.2 Scope

The scope of determining whether modifications need to be considered starts with identification of situations where *Sample* collection involves *Athletes* with disabilities and ends with the necessary modifications to *Sample* collection procedures and equipment as possible for these *Athletes*.

B.3 Responsibility

The *ADO* has responsibility for ensuring, when possible, that the *DCO* has any information and *Sample Collection Equipment* necessary to conduct a *Sample Collection Session* with an *Athlete* with a disability. The *DCO* has responsibility for the *Sample* collection.

B.4 Requirements

B.4.1 All aspects of notification and *Sample* collection for *Athletes* with disabilities shall be carried out in accordance with the standard notification and *Sample* collection procedures unless modifications are necessary due to the *Athlete's* disability.

B.4.2 In planning or arranging *Sample* collection, the *ADO* and *DCO* shall consider whether there will be any *Sample* collection for *Athletes* with disabilities that may require modifications to the standard procedures for notification or *Sample* collection, including *Sample Collection Equipment* and facilities.

B.4.3 The *DCO* shall have the authority to make modifications as the situation requires when possible and as long as such modifications will not compromise the identity, security or integrity of the *Sample*.

B.4.4 For *Athletes* with a physical disability or a sensorial disability, the *Athlete* can be assisted by the *Athlete's* representative or *Sample Collection Personnel* during the *Sample Collection Session* where authorised by the *Athlete* and agreed to by the *DCO*.

B.4.5 For *Athletes* with an intellectual disability, the *ADO* or *DCO* shall determine whether the *Athlete* must have a representative at the *Sample Collection Session* and the nature of the assistance that the representative must provide. Additional assistance can be provided by the representative or *Sample Collection Personnel* during the *Sample Collection Session* where authorised by the *Athlete* and agreed to by the *DCO*.

B.4.6 The DCO can decide that alternative Sample Collection Equipment or facilities will be used when required to enable the *Athlete* to provide the *Sample* as long as the *Sample's* identity, security and integrity will not be affected.

B.4.7 *Athletes* who are using urine collection or drainage systems are required to eliminate existing urine from such systems before providing a urine *Sample* for analysis.

B.4.8 The DCO will record modifications made to the standard *Sample* collection procedures for *Athletes* with disabilities, including any applicable modifications specified in the above actions.

Annex C - Collection of urine *Samples*

C.1 Objective

To collect an *Athlete's* urine *Sample* in a manner that ensures:

- a) Consistency with relevant principles of internationally recognised standard precautions in healthcare settings so that the health and safety of the *Athlete* and Sample Collection Personnel are not compromised;
- b) The *Sample* is of a quality and quantity that meets laboratory guidelines;
- c) The *Sample* is clearly and accurately identified; and
- d) The *Sample* is securely sealed.

C.2 Scope

The collection of a urine *Sample* begins with ensuring the *Athlete* is informed of the *Sample* collection requirements and ends with discarding any residual urine remaining at the end of the *Athlete's* Sample Collection Session.

C.3 Responsibility

The DCO has the responsibility for ensuring that each *Sample* is properly collected, identified and sealed. The DCO/Chaperone has the responsibility for directly witnessing the passing of the urine *Sample*.

C.4 Requirements

C.4.1 The DCO shall ensure that the *Athlete* is informed of the requirements of the *Sample* collection, including any modifications as provided for in Annex B – Modifications for *Athletes* with disabilities.

C.4.2 The DCO shall ensure that the *Athlete* is offered a choice of appropriate equipment for collecting the *Sample*. If the nature of an *Athlete's* disability requires that he/she must use additional or other equipment as provided for in Annex B – Modifications for *Athletes* with disabilities, the DCO shall inspect that equipment to ensure that it will not affect the identity or integrity of the *Sample*.

C.4.3 The DCO shall instruct the *Athlete* to select a collection vessel.

C.4.4 When the *Athlete* selects a collection vessel and for selection of all other Sample Collection Equipment that directly holds the urine *Sample*, the DCO will instruct the *Athlete* to check that all seals on the selected equipment are intact and the equipment has not been tampered with. If the *Athlete* is not satisfied with the selected equipment, he/she may select another. If the *Athlete* is not satisfied with any of the equipment available for the selection, this shall be recorded by the DCO.

If the DCO does not agree with the *Athlete's* opinion that all of the equipment available for the selection is unsatisfactory, the DCO shall instruct the *Athlete* to proceed with the Sample Collection Session. If the DCO agrees with the reasons put forward by the *Athlete* that all of the equipment available for the selection is unsatisfactory, the DCO shall terminate the collection of the *Athlete's* urine *Sample* and this shall be recorded by the DCO.

C.4.5 The *Athlete* shall retain control of the collection vessel and any *Sample* provided until the *Sample* is sealed, unless assistance is required by an *Athlete's* disability as provided for in Annex B – Modifications for *Athletes* with disabilities.

C.4.6 The DCO/Chaperone who witnesses the passing of the *Sample* shall be of the same gender as the *Athlete* providing the *Sample*.

C.4.7 The DCO/Chaperone and *Athlete* shall proceed to an area of privacy to collect a *Sample*.

C.4.8 The DCO/Chaperone shall witness the *Sample* leaving the *Athlete's* body and record the witnessing in writing.

C.4.9 The DCO shall use the relevant laboratory's specifications to verify, in full view of the *Athlete*, that the volume of the urine *Sample* satisfies the laboratory's requirements for analysis.

C.4.10 Where the volume of urine is insufficient, the DCO shall conduct a partial *Sample* collection procedure as prescribed in Annex E – Urine *Samples* – insufficient volume.

C.4.11 The DCO shall instruct the *Athlete* to select a *Sample* collection kit containing A and B bottles in accordance with C.4.4.

C.4.12 Once a *Sample* collection kit has been selected, the DCO and the *Athlete* shall check that all code numbers match and that this code number is recorded accurately by the DCO.

If the *Athlete* or DCO finds that the numbers are not the same, the DCO shall instruct the *Athlete* to choose another kit in accordance with C.4.4. The DCO shall record the matter.

C.4.13 The *Athlete* shall pour the relevant laboratory's prescribed minimum volume of urine into the B bottle, and then fill the A bottle as much as possible. The *Athlete* shall then fill the B bottle as much as possible with the remaining urine. The *Athlete* shall ensure that a small amount of urine is left in the collection vessel.

C.4.14 The *Athlete* shall seal the bottles as directed by the DCO. The DCO shall check, in full view of the *Athlete*, that the bottles have been properly sealed.

C.4.15 The DCO shall use the relevant laboratory's guidelines for pH and specific gravity to test the residual urine in the collection vessel to determine if the *Sample* is likely to meet the laboratory guidelines. If it is

not, then the DCO shall follow Annex F - Urine *Samples* - *Samples* that do not meet laboratory pH and specific gravity guidelines.

C.4.16 The DCO shall ensure any residual urine that will not be sent for analysis is discarded in full view of the *Athlete*.

Annex D - Collection of blood Samples

D.1 Objective

To collect an *Athlete's* blood *Sample* in a manner that ensures:

- a) The health and safety of the *Athlete* and Sample Collection Personnel are not compromised;
- b) The *Sample* is of a quality and quantity that meets the relevant analytical guidelines;
- c) The *Sample* is clearly and accurately identified; and
- d) The *Sample* is securely sealed.

D.2 Scope

The collection of a blood *Sample* begins with ensuring the *Athlete* is informed of the *Sample* collection requirements and ends with properly storing the *Sample* prior to dispatch for analysis at the WADA accredited laboratory or as otherwise approved by WADA.

D.3 Responsibility

D.3.1 The DCO has the responsibility for ensuring that:

- a) Each *Sample* is properly collected, identified and sealed; and
- b) All *Samples* have been properly stored and dispatched in accordance with the relevant analytical guidelines.

D.3.2 The Blood Collection Official has the responsibility for collecting the blood *Sample*, answering related questions during the provision of the *Sample*, and proper disposal of used blood sampling equipment not required for completing the Sample Collection Session.

D.4 Requirements

D.4.1 Procedures involving blood shall be consistent with relevant principles of internationally recognised standard precautions in health care settings.

D.4.2 Blood Sample Collection Equipment shall consist of, either an A sample tube, or an A sample tube and a B sample tube. If the sample collection consists solely of blood then a B sample shall be collected and used as a confirmation if required.

D.4.3 The DCO shall ensure that the *Athlete* is informed of the requirements of the *Sample* collection, including any modifications as provided for in Annex B – Modifications for *Athletes* with disabilities.

D.4.4 The DCO/Chaperone and *Athlete* shall proceed to the area where the *Sample* will be provided.

D.4.5 The DCO shall ensure the *Athlete* is offered comfortable conditions including being in a relaxed position for at least 10 minutes prior to providing a *Sample*.

D.4.6 The DCO shall instruct the *Athlete* to select the *Sample* collection kit/s required for collecting the *Sample* and to check that the selected equipment has not been tampered with and the seals are intact. If the *Athlete* is not satisfied with a selected kit, he/she may select another. If the *Athlete* is not satisfied with any kits and no others are available, this shall be recorded by the DCO.

If the DCO does not agree with the *Athlete's* opinion that all of the available kits are unsatisfactory, the DCO shall instruct the *Athlete* to proceed with the *Sample Collection Session*.

If the DCO agrees with the reasons put forward by the *Athlete* that all available kits are unsatisfactory, the DCO shall terminate the collection of the *Athlete's* blood *Sample* and this shall be recorded by the DCO.

D.4.7 When a *Sample* collection kit has been selected, the DCO and the *Athlete* shall check that all code numbers match and that this code number is recorded accurately by the DCO.

If the *Athlete* or DCO finds that the numbers are not the same, the DCO shall instruct the *Athlete* to choose another kit in accordance with D.4.5. The DCO shall record the matter.

D.4.8 The Blood Collection Official shall clean the skin with a sterile disinfectant wipe or swab in a location unlikely to adversely affect the *Athlete* or his/her performance and, if required, apply a tourniquet. The Blood Collection Official shall take the blood *Sample* from a superficial vein into the final collection container. The tourniquet, if applied, shall be immediately removed after the venipuncture has been made.

D.4.9 The amount of blood removed shall be adequate to satisfy the relevant analytical requirements for the *Sample* analysis to be performed.

D.4.10 If the amount of blood that can be removed from the *Athlete* at the first attempt is insufficient, the Blood Collection Official shall repeat the procedure. Maximum attempts shall be three. Should all attempts fail, then the Blood Collection Official shall inform the DCO. The DCO shall terminate the collection of the blood *Sample* and record this and the reasons for terminating the collection.

D.4.11 The Blood Collection Official shall apply a dressing to the puncture site/s.

D.4.12 The Blood Collection Official shall dispose of used blood sampling equipment not required for completing the *Sample Collection Session*.

D.4.13 The *Athlete* shall seal his/her *Sample* into the *Sample* collection kit as directed by the DCO. In full view of the *Athlete*, the DCO shall check that the sealing is satisfactory.

D.4.14 The sealed *Sample* shall be kept at a cool, but not freezing, temperature prior to analysis at the Doping Control Station or dispatch for analysis at the *WADA* accredited laboratory or as otherwise approved by *WADA*.

Annex E - Urine *Samples* - Insufficient volume

E.1 Objective

To ensure that where an insufficient volume of urine is provided, appropriate procedures are followed.

E.2 Scope

The procedure begins with informing the *Athlete* that the *Sample* is of insufficient volume and ends with the provision of a *Sample* of sufficient volume.

E.3 Responsibility

The DCO has the responsibility for declaring the *Sample* volume insufficient and for collecting the additional *Sample/s* to obtain a combined *Sample* of sufficient volume.

E.4 Requirements

E.4.1 If the *Sample* collected is of insufficient volume, the DCO shall inform the *Athlete* that a further *Sample* shall be collected to meet the relevant laboratory's volume requirements.

E.4.2 The DCO shall instruct the *Athlete* to select partial *Sample Collection Equipment* in accordance with C.4.4.

E.4.3 The DCO shall then instruct the *Athlete* to open the relevant equipment, pour the insufficient *Sample* into the container and seal it as directed by the DCO. The DCO shall check, in full view of the *Athlete*, that the container has been properly sealed.

E.4.4 The DCO and the *Athlete* shall check that the equipment code number, and the volume and identity of the insufficient *Sample* are recorded accurately by the DCO. Either the *Athlete* or the DCO shall retain control of the sealed partial *Sample*.

E.4.5 While waiting to provide an additional *Sample*, the *Athlete* shall remain under continuous observation and be given the opportunity to hydrate.

E.4.6 When the *Athlete* is able to provide an additional *Sample*, the procedures for collection of the *Sample* shall be repeated as prescribed in Annex C – Collection of urine *Samples* until a sufficient volume of urine will be provided by combining the initial and additional *Sample/s*.

E.4.7 When the DCO is satisfied that a sufficient volume of urine has been provided, the DCO and *Athlete* shall check the integrity of the seal/s on the partial *Sample* container/s containing the previously provided insufficient *Sample/s*. Any irregularity with the integrity of the seal/s will

be recorded by the DCO and investigated according to Annex A – Investigating a possible failure to comply.

E.4.8 The DCO shall then direct the *Athlete* to break the seal/s and combine the *Samples*, ensuring that additional *Samples* are added sequentially to the first *Sample* collected until the required volume is met.

E.4.9 The DCO and *Athlete* shall then continue with C.4.11.

Annex F - Urine Samples - Samples that do not meet laboratory pH or specific gravity guidelines

F.1 Objective

To ensure that when the urine *Sample* does not meet the contracted laboratory pH or specific gravity guidelines, appropriate procedures are followed.

F.2 Scope

The procedure begins with the DCO informing the *Athlete* that a further *Sample* is required and ends with the collection of a *Sample* that meets laboratory pH and specific gravity guidelines or appropriate follow-up action by the *ADO* if required.

F.3 Responsibility

The *ADO* is responsible for establishing criteria for the number of additional *Samples* to be collected at the *Athlete's Sample Collection Session*. If the additional *Sample/s* collected do not meet the relevant laboratory's guidelines for analysis, the *ADO* is responsible for scheduling a new *Sample Collection Session* for the *Athlete* and, if required, taking subsequent appropriate action.

The DCO is responsible for collecting additional *Sample/s* in accordance with the *ADO's* criteria.

F.4 Requirements

F.4.1 The *ADO* shall establish criteria for the number of additional *Samples* to be collected by the DCO when the DCO determines that an *Athlete's Sample* is unlikely to meet the relevant laboratory's pH or specific gravity guidelines.

F.4.2 The DCO shall inform the *Athlete* that he/she is required to provide a further *Sample*.

F.4.3 While waiting to provide an additional *Sample*, the *Athlete* shall remain under continuous observation.

F.4.4 When the *Athlete* is able to provide an additional *Sample*, the DCO shall repeat the procedures for collection of the *Sample* as prescribed in Annex C – Collection of urine *Sample* and in accordance with the *ADO's* criteria for the number of additional *Samples* to be collected as established in F.4.1..

F.4.5 The DCO shall record that the *Samples* collected belong to a single *Athlete* and the order in which the *Samples* were provided.

F.4.6 The DCO shall then continue with C.4.16.

F.4.7 If it is determined by the relevant laboratory that all of the *Athlete's Samples* do not meet the laboratory's pH and specific gravity requirements for analysis and this is not related to natural causes, the ADO shall schedule another Sample Collection Session for the *Athlete* as *Target Testing* as soon as possible.

F.4.8 If the *Target Testing Sample Collection Session* also results in *Samples* that do not meet the laboratory's pH and/or specific gravity requirements for analysis, the ADO shall investigate a possible anti-doping rule violation.

Annex G - Sample Collection Personnel Requirements

G.1 Objective

To ensure that Sample Collection Personnel have no conflict of interest and have adequate qualifications and experience to conduct *Sample* collection sessions.

G.2 Scope

Sample Collection Personnel requirements starts with the development of the necessary competencies for Sample Collection Personnel and ends with the provision of identifiable accreditation.

G.3 Responsibility

The *ADO* has the responsibility for all activities defined in this Annex G.

G.4 Requirements - Qualifications and Training

G.4.1 The *ADO* shall determine the necessary competence and qualification requirements for the positions of Doping Control Officer, Chaperone and Blood Collection Official. The *ADO* shall develop duty statements for all Sample Collection Personnel that outline their respective responsibilities. As a minimum:

- a) Sample Collection Personnel shall be of adult age.
- b) Blood Collection Officials shall have adequate qualifications and practical skills required to perform blood collection from a vein.

G.4.2 The *ADO* shall ensure that Sample Collection Personnel that have an interest in the outcome of the collection or testing of a *Sample* from any *Athlete* who might provide a *Sample* at a session are not appointed to that *Sample* collection session. Sample Collection Personnel are deemed to have an interest in the collection of a *Sample* if they are:

- a) Involved in the planning of the sport for which testing is being conducted; or
- b) Related to, or involved in the personal affairs of any *Athlete* who might provide a *Sample* at that session.

G.4.3 The *ADO* shall establish a system that ensures that Sample Collection Personnel are adequately qualified and trained to carry out their duties.

G.4.4 The training program for Chaperones and Blood Collection Officers as a minimum shall include studies of all relevant requirements of the testing process and familiarization of relevant standard precautions in healthcare settings.

G.4.5 The training program for Doping Control Officers as a minimum shall include:

- a) Comprehensive theoretical training in different types of testing activities relevant to the Doping Control Officer position;
- b) One observation of all doping control activities related to requirements in this standard, preferably on site;
- c) The satisfactory performance of one complete *Sample* collection on site under observation by a qualified Doping Control Officer or similar. The requirement related to actual passing of *Sample* shall not be included in the on site observations.

G.4.6 The ADO shall maintain records of education, training, skills and experience.

G.5 Requirements - Accreditation, re-accreditation and delegation

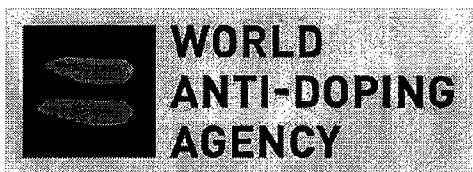
G.5.1 The ADO shall establish a system for accrediting and re-accrediting Sample Collection Personnel.

G.5.2 The ADO shall ensure that Sample Collection Personnel have completed the training program and are familiar with the requirements in this testing standard before granting accreditation.

G.5.3 Accreditation shall only be valid for a maximum of two years. Sample Collection Personnel shall be required to repeat a full training program if they have not participated in *Sample* collection activities within the year prior to re-accreditation.

G.5.4 Only Sample Collection Personnel that have an accreditation recognised by the ADO shall be authorised by the ADO to conduct *Sample* collection activities on behalf of the ADO.

G.5.5 Doping Control Officers may personally perform any activities involved in the Sample Collection Session, with the exception of blood collection unless particularly qualified, or they may direct a Chaperone to perform specified activities that fall within the scope of the Chaperone's authorised duties.



World Anti-Doping Program

GUIDELINES FOR URINE SAMPLE COLLECTION

Version 4
June 2004

WADA STANDARDS AND HARMONIZATION – GUIDELINE FOR URINE SAMPLE COLLECTION

INDEX

| | <u>Page</u> |
|--|-------------|
| 1. <u>Objective</u> | 3 |
| 2. <u>Scope</u> | 3 |
| 3. <u>Responsibility</u> | |
| 3.1 <i>Doping Control Officer</i> | 3 |
| 3.2 <i>Chaperone</i> | 3 |
| 3.3 <i>Athlete</i> | 4 |
| 3.4 <i>Athlete Representative</i> | 4 |
| 4. <u>Definitions</u> | 4 |
| 5. <u>Protocol for the Urine Sample Collection Session</u> | |
| 5.1 <i>Brief personnel on roles and responsibilities</i> | 6 |
| 5.2 <i>Assess the facilities</i> | 6 |
| 5.3 <i>Prepare the necessary equipment.</i> | 7 |
| 5.4 <i>Athlete selection</i> | 8 |
| 5.5 <i>Athlete notification</i> | 8 |
| 5.6 <i>Escorting the Athlete to the Doping Control Station</i> | 10 |
| 5.7 <i>Arrival at the Doping Control Station</i> | 11 |
| 5.8 <i>Selection of the sample collection vessel</i> | 12 |
| 5.9 <i>Sample provision</i> | 13 |
| 5.10 <i>Insufficient volume</i> | 14 |
| 5.11 <i>Dividing and sealing the sample</i> | 15 |
| 5.12 <i>Samples not meeting laboratory guidelines for analysis</i> | 16 |
| 5.13 <i>Paperwork</i> | 17 |
| 5.14 <i>Sample storage</i> | 18 |
| 5.15 <i>Transport of samples</i> | 18 |
| 5.16 <i>Hand-over of samples to the laboratory.</i> | 19 |
| 6. <u>Modifications for Minors and Athletes with a Disability</u> | |
| 6.1 <i>Minors</i> | 19 |
| 6.2 <i>Athletes with restricted mobility/restricted manual dexterity</i> | 19 |
| 6.3 <i>Athletes with visual impairment</i> | 19 |
| 6.4 <i>Athletes with an intellectual disability</i> | 20 |
| 6.5 <i>Athletes using condom drainage/indwelling catheter drainage</i> | 20 |
| 6.6 <i>Athletes who self-catheterize</i> | 20 |

Appendix 1 Chaperone Training Guidelines

1. Objective

This guideline expands upon the International Standard for Testing and details the recommended process for the collection of urine for doping control purposes, both In-Competition and Out-of-Competition. The guideline includes on-site preparation, sample collection and post-test administration.

With the exception of those mandatory areas which are part of the World Anti-Doping Program, the processes outlined in this document are not mandatory, but are aimed at assisting *Anti-Doping Organizations* in the development of systems and protocols for urine sample collection. The method of sample collection may vary from these recommendations in some circumstances; however, minimum standards should apply to ensure that the integrity of the sample is maintained.

2. Scope

This Guideline begins with the arrival of *Sample Collection Personnel* at the *Doping Control Station*, and ends with the dispatch of the urine sample to the laboratory.

3. Responsibility

3.1. *Doping Control Officer (DCO)*

(One lead/senior DCO shall take responsibility for sample collection services)

- Organize and brief *Sample Collection Personnel*.
- Ensure that Chaperones are trained in carrying out relevant activities.
- Liaise with sport representatives, if relevant.
- Organize equipment, including all relevant documentation.
- Assess and organize the facilities.
- Arrange or perform notification and escorting of *Athletes*.
- Ensure that the *Athlete's* rights and responsibilities are explained.
- Explain, or arrange explanation of, the process for urine sample collection to *Athletes* and Athlete Representatives, as necessary.
- Witness, or arrange the witnessing of, sample provision.
- Co-ordinate collection of accompanying blood sample if necessary.
- Complete, or arrange completion of, and verify, the relevant paperwork.
- Verify the chain of custody.
- Organize courier services, if necessary.

3.2. *Chaperone*

- Notify the *Athlete* in person as instructed by the DCO.
- Escort the *Athlete* from notification until arrival at the *Doping Control Station*.
- If appropriately trained and authorized (see Appendix 1), act as the Witness for sample provision as instructed by the DCO and complete the relevant section of the doping control documentation as instructed by the DCO.

- NOTE: Some *Anti-Doping Organizations* prefer that Chaperones do not witness sample provision but conduct *Athlete* notification only. These Guidelines allow for both these scenarios.

3.3. *Athlete*

- Request the presence of an Athlete Representative, if desired.
- Report for doping control as soon as possible, and within the specified time frame.
- Be escorted from notification to sample provision.
- Be responsible for any food or beverage consumed prior to sample provision.
- Be familiar with the sample collection process.
- Be responsible at all times for his/her own sample(s) from provision to final sealing.
- Observe the procedure and ensure there are no irregularities.
- Declare any medications as specified on the doping control documentation.
- Provide a TUE certificate if applicable.
- Make comments relating to the sample collection process on the doping control documentation, if applicable.
- Sign documentation as requested by the DCO.

3.4. Athlete Representative (*presence optional, at Athlete's request*)

- Accompany the *Athlete* during notification.
- Accompany the *Athlete* to the *Doping Control Station*.
- Assist in the selection of equipment and the sealing process where asked to do so by the *Athlete*.
- Assist the *Athlete* in the completion of paperwork where asked to do so by the *Athlete*.
- Be familiar with the sample collection process.
- Observe the sample collection process and ensure there are no irregularities.
- Sign documentation as requested by the DCO.

4. Definitions

"*Anti-Doping Organization*" means a Signatory (of the World Anti-Doping Code) that is responsible for adopting rules, for initiating, implementing or enforcing any part of the doping control process. This includes, for example, the International Olympic Committee, the International Paralympic Committee, or other major event organizations that conduct testing at their events, WADA, International Federations, and National Anti-Doping Organizations.

'*Athlete*' means for purposes of doping control, any person who participates in sport at the international level (as defined by each International Federation), or national level (as defined by each National Anti-Doping Organization) and any additional person who participates in sport at the lower level if designated by the person's National Anti-Doping Organization. For purposes of anti-doping information and education, any person who participates in sport under the authority of any signatory, government, or other sports organization accepting the Code.

'Athlete Representative' means a person designated by the *Athlete* to assist with the verification of the sample collection procedure, (not including the passing of the sample). This person may be a member of the *Athlete's* support personnel, such as a coach or team doctor, a family member, or other.

'Chaperone' means an official who is trained and authorized by the ADO to carry out specific duties including notification of the *Athlete* selected for sample collection, escorting and observing the *Athlete* until arrival at the *Doping Control Station*, and/or witnessing and verifying the provision of the sample where training qualifies him/her to do so.

'Doping Control Officer' means an official who has been trained and authorized by the ADO with delegated responsibility for the on-site management of a sample collection session.

'Doping Control Station' means the location where the *urine sample collection session* will be conducted.

'In-Competition' means for purposes of differentiating between *In Competition* and *Out of Competition* Testing, unless provided otherwise in the rules of an ADO, an *In-Competition* test is a test where an *Athlete* is selected for testing in connection with a specific competition.

'Minor' means a natural person who has not reached the age of maturity as established by the applicable laws of his or her country of residence.

'Out of Competition' means any doping control which is not *In-Competition*.

'No-Advance-Notice' means a doping control which takes place with no advance warning to the *Athlete*, and where the *Athlete* is continuously Chaperoned from the moment of notification through sample provision.

'TUE' means a Therapeutic Use Exemption (see International Standard for Therapeutic Use Exemptions).

'Sample Collection Personnel' is a collective term for qualified officials authorized by the ADO who may carry out or assist with duties during the sample collection session.

'Urine Sample Collection Session' means the sequential activities that directly involve the *Athlete* from notification until the *Athlete* leaves the *Doping Control Station* having provided his/her samples(s).

'Weighted Selection' means a ranking method of selecting *Athletes* using criteria where the ranking is based on the potential risk of doping and possible doping patterns.

'Witness' means the member of *Sample Collection Personnel* who observes the passing of the sample by the *Athlete* in accordance with the procedures for observation.

5. Protocol for the Urine Sample Collection Session

The protocol for the *urine sample collection session* is divided into the following steps:

5.1. Brief personnel on roles and responsibilities

- 5.1.1. The Lead DCO shall brief the *Sample Collection Personnel* on their roles and responsibilities prior to or upon arrival at the *Doping Control Station*. This will include *Athlete* notification, escorting, urine sample collection, and related blood sample collection if applicable.
- 5.1.2. In the case of a team which includes Chaperones with no experience, the DCO shall train the Chaperones on-site. Such training shall include the requirements for notification, escorting and witnessing sample provision, as well as confidentiality obligations (see Appendix 1 for Chaperone Training Guidelines).
- 5.1.3. The DCO shall provide required documentation such as proof of authority to conduct sample collection, to the *Sample Collection Personnel* if applicable.

5.2 Assess the facilities

- 5.2.1 The minimum requirements to be met to enable use of a facility as a *Doping Control Station* are privacy and sole-use. If the facility does not offer the *Athlete* privacy, and/or is intended to be used for purposes other than doping control whilst sample collection is being carried out, the Lead DCO may decide not to proceed with testing. The reasons for such a decision must be documented.
- 5.2.2 For *In-Competition* testing, where possible, the *Doping Control Station* should meet the following criteria:
 - Be solely reserved for doping control purposes
 - Maintain *athlete* privacy and confidentiality
 - Be accessible only to authorized personnel
 - Be secure enough to store sample collection equipment
 - Be comprised of a waiting area with chairs and a separate administration area with a table and chairs for completion of paperwork. There should be adjoining toilet facilities for sample provision, which should ideally consist of cubicles large enough for the Witness and the *Athlete*.
 - Include facilities to allow the *Athlete* to wash his/her hands.
 - Be large enough to accommodate the number of *Athletes*, Athlete Representatives and *Sample Collection Personnel* who will occupy the area
 - Be suitably located in relation to the field of play or other location where *Athletes* will be notified.

- 5.2.3 For *Out-of-Competition* testing, the facilities used should, where possible, provide a suitable environment for waiting and administration, and afford the *Athlete* privacy.

NOTE: Although the term '*Doping Control Station*' is also used for *Out-of-Competition* testing, this facility might be an *athlete's* home or a hotel room, rather than an officially designated facility for doping control.

- 5.2.4 The *Doping Control Station* at an event should also contain a selection of sealed, non-alcoholic, caffeine-free drinks for *Athletes*.
- 5.2.5 Access to the *Doping Control Station* is restricted to the *Athlete*, the Athlete Representative, an interpreter if required, and *Sample Collection Personnel*, unless otherwise agreed by the Lead DCO. Additional personnel requesting access may include an IF representative, an ADO observer, an auditor or a WADA Independent Observer. These personnel shall have adequate authorization available for the Lead DCO to review upon arrival at the *Doping Control Station*.
- 5.2.6 The Lead DCO may wish to assign a member of the sample collection team to monitor access to the *Doping Control Station*, and ensure that only authorized persons are admitted.
- 5.2.7 Members of the media must not be allowed to enter the *Doping Control Station* at any time.
- 5.3 *Prepare the necessary equipment*
- 5.3.1 The DCO shall ensure equipment supplies are adequate for the sample collection session. The type of equipment may vary but, as a guideline, will include:

- Sealed, sterile urine collection vessels.
- Partial sample kits.
- Equipment for measuring pH (if required), and specific gravity.
- Sealed, tamper-evident containers for A and B samples.
- Sealed, tamper-evident transport containers (if applicable).
- Secure transport bags.
- Disposable gloves (optional).
- Soap or hand wash
- Paper towels
- Garbage bin or similar for disposal
- Individually sealed, non-caffeinated and non-alcoholic beverages
- All doping control documentation, including doping control forms, *Athlete* notification forms, supplementary report forms, chain of custody forms, etc.

5.3.2 Any sample collection equipment systems used shall meet the following minimum criteria:

- Have a unique numbering system incorporated into all containers in which the *Athlete's* sample is sealed.
- Have a sealing system that is tamper-evident.
- Ensure the identity of the *Athlete* is not evident from the equipment itself.
- Ensure that all equipment is clean and sealed prior to use.

5.4 *Athlete Selection*

5.4.1 The DCO will select *athletes* according to the selection policy indicated by the ADO. This may include one or all of the following: target testing (named *athletes* or categories), *weighted* selection and random selection.

5.4.2 In the case of random selection, the ADO/DCO may choose to use one of the following selection criteria. The criteria chosen shall be appropriate for the sport, e.g:

- Finishing position
- Vest/jersey number
- Entry number
- Lane number
- Any other fair and transparent criteria for selection

5.4.3 Once the criteria has been determined, the actual selection method may be one of the following:

- Numbered cards placed face-down on a table
- Random draw of numbers (or names) from a closed container such as a cloth bag
- Use of an electronic random number generator
- Any other fair and transparent method of selection

In order to provide transparency and accountability, random selection made in the field may be witnessed by a coach or sporting official, or can be shown to the selected athlete if requested. For example a signature on the back of numbered cards.

5.4.4 Following the selection of the *Athlete*, the Lead DCO shall ensure that selection decisions are disclosed on a need-to-know basis only to ensure that testing is *No-Advance Notice*.

5.5 *Athlete notification*

5.5.1 The DCO/Chaperone shall establish the location of the selected *Athlete*, and plan the approach and timing of notification, taking into account any specific circumstances such as the competition/training schedule, and such that the notification will be carried out as *No-Advance-Notice* notification.

- 5.5.2 The DCO/Chaperone shall identify him/herself and shall show the *Athlete* the official card/document naming the ADO which has granted the authority to test. Additional identification proving affiliation to the authorized sample collection authority shall also be provided, if this authority is not the ADO which authorized the test. DCO identification documents shall include name, photograph, and the documents' expiry date. Chaperone identification documents shall ideally also include name, photograph, and the documents' expiry date, and as a minimum shall comprise a dated document naming them as an authorized member of the sample collection team, which they shall show to the athlete in conjunction with a piece of photo ID.
- 5.5.3 The DCO/Chaperone shall, at a minimum, verbally confirm the *Athlete's* identity. If the *Athlete* is carrying photo ID, this may be checked at this stage. An *Athlete's* inability to provide photo ID shall not invalidate a test. Formal identification can be established by starting number, accreditation, third party witness, if the *Athlete* is known to the DCO/Chaperone, or other viable method. If the *Athlete's* identity is unknown and can not be established in any manner, the DCO must contact the ADO for further instructions.
- 5.5.4 The DCO/Chaperone shall show the *Athlete* the notification form (which may be part of the doping control form), and notify the *Athlete* of his/her selection for testing, the authority under which sample collection is to be conducted, and the requirement to provide a urine (and blood, if applicable) sample, and shall inform the *Athlete* of the following rights and responsibilities:
- a) For all types of testing
 - The right to have a representative and, if required, an interpreter present.
 - The right to ask for additional information about the sample collection process
 - The possible consequence of an anti-doping rule violation for failing to submit to sample collection.
 - The requirement to remain in sight of the designated DCO/Chaperone until completion of the sample collection procedure.
 - The requirement to bring satisfactory identification to the *Doping Control Station* if this has not already been provided.
 - b) For the purpose of *In-Competition Testing*
 - The requirement to report to the *Doping Control Station* as soon as possible and not later than 60 minutes from notification.
 - The DCO shall consider reasonable requests to delay the reporting time, if a Chaperone is available, to enable the *Athlete* to complete one or more of the following:
 - locate a representative and/or interpreter
 - warm down
 - attend a medal ceremony
 - participate in further events

- fulfill media commitments
 - receive treatment for injury
 - any other reason accepted by the DCO
- The DCO shall document reasons for any delay that may require further investigation by the ADO.
- c) For the purpose of *Out of Competition Testing*
- The requirement to report to the doping control station and commence sample provision as soon as possible. The DCO shall consider requests to delay reporting, if a Chaperone is available, to enable the *Athlete* to complete one or more of the following:
 - locate a representative, if available
 - complete a training session
 - receive treatment for injury
 - any other reason accepted by the DCO
 - The DCO shall document reasons for any delay that may require further investigation by the ADO.
- 5.5.5 If a selected *athlete* is not located based on available information, the DCO shall attempt to locate the *athlete* by other means, but ensure that *No-Advance-Notice* notification is used as a notification method. The DCO shall notify the ADO for further instructions if the *athlete* is not located. (See WADA's No-Advance Notice Testing Guidelines).
- 5.5.6. The DCO shall report any decision to the ADO if an unexpected situation arises requiring the notification to become advance notice.
- 5.5.7. The *Athlete* shall read and sign the *Athlete* notification form or doping control form as directed by the DCO/Chaperone.
- 5.5.8. If an *Athlete* copy of the official notification record exists, this will be given to the *Athlete*.
- 5.5.9 If the *Athlete* refuses to sign that he/she has been notified, or evades notification, the DCO/Chaperone shall make all reasonable attempts to persuade the *Athlete* to comply, including informing the *Athlete* again that failure to comply may result in sanction for an anti-doping rule violation. If the *Athlete* continues to refuse, the Chaperone must report this to the Lead DCO immediately, and the DCO shall attempt to notify the *Athlete*. If the *Athlete* still refuses to be notified, the DCO shall document the facts, including the reasons for refusal given by the *Athlete*. The DCO shall endeavor to obtain witness signatures to confirm the *Athlete's* refusal, and shall contact the ADO for further instructions as soon as possible.

5.6 *Escorting the Athlete to the Doping Control Station.*

- 5.6.1 The DCO/Chaperone shall ensure that the *Athlete* is escorted from the place of notification to the *Doping Control Station* under constant supervision.
- 5.6.2 The DCO/Chaperone shall discourage the *Athlete* from taking a bath or shower, and shall ensure he/she does not urinate prior to reporting at the *Doping Control Station*. The first urine sample post notification shall be collected.
- 5.6.3 The DCO/Chaperone can not prevent the *Athlete* eating or drinking products of their choice, but shall recommend that the *Athlete* chooses from a selection of individually sealed, non-caffeinated and non-alcoholic beverages in order to hydrate. The DCO/Chaperone shall not handle food or drink items for the *Athlete*.
- 5.6.4 The DCO/Chaperone shall escort the *Athlete* at all times until the sample collection procedures have been completed, or shall ensure that another DCO/Chaperone has taken over escorting the *Athlete*.
- 5.6.5 The Chaperone shall inform the Lead DCO as soon as practical without leaving the *Athlete* unattended, and ensuring discretion, of any irregularities in notification and/or during the observation period. Irregularities shall be documented by the Lead DCO if relevant.

NOTE: The ADO is responsible for establishing guidelines for what constitutes suspicious *athlete* behavior – examples might be; evading observation, ingesting an unidentified substance, a distressed call to a coach or other unusual behavior.

- 5.6.6 If an *Athlete* notified of an advance notice sample collection does not report to the *Doping Control Station* at the designated time, the DCO shall use his/her judgement as to whether to attempt to contact the *athlete*. At a minimum, the DCO shall wait 30 minutes after the appointed time before departing.
- 5.6.7 If an *Athlete* reports to the *Doping Control Station* after the minimum waiting time, and prior to the DCO's departure, the Lead DCO shall decide whether to process a possible failure to comply. Where possible, the DCO shall proceed with collecting a sample, and shall document the details of the delay.

NOTE: *No-Advance-Notice* is the preferred method of doping control. The situation described in 5.6.6 and 5.6.7 shall be in exceptional circumstances only; an *Athlete* should ideally be escorted at all times, and the escorting DCO/Chaperone shall ensure that the *Athlete* reports for doping control as quickly as possible, taking into account the provisions of 5.5.4.

5.7 *Arrival at the Doping Control Station*

- 5.7.1 The *Athlete* arrives at the *Doping Control Station* with a DCO/Chaperone and, if requested, an Athlete Representative and/or interpreter. At this time, the *Athlete* should present photo ID to the DCO. An *Athlete's* inability to provide

photo ID shall not invalidate a test. Alternative methods of *Athlete* identification are outlined in 5.5.3.

- 5.7.2 An entry and exit log shall be maintained to record the names of the persons entering facility, their position, and the times of arrival and departure.
- 5.7.3 The *Athlete* shall be provided with the opportunity to hydrate.
- 5.7.4 If the *Athlete* is providing a blood sample at the same session, the DCO may request that the *Athlete* provide the blood sample first.
- 5.7.5 Irrespective of the testing type or escorting requirements prior to the time of arrival, once the *athlete* has arrived at the *Doping Control Station* he/she must be under observation at all times until sample collection is completed
- 5.7.6 The *Athlete* may request to leave the *Doping Control Station* for a time, for reasons defined in 5.5.4. The *Athlete* must be escorted continuously at such times, and the purpose of leaving, agreed time of return, and actual time of return shall be documented by the Lead DCO. If a Chaperone is not available, the DCO shall ask the *Athlete* to remain in the *Doping Control Station*. If an *Athlete* insists on leaving the *Doping Control Station*, the circumstances shall be documented by the Lead DCO.
- 5.7.7 Before sample collection, the DCO shall ask the *Athlete* whether they have been tested before, and whether they require an explanation of the collection procedure.
- 5.7.8 If the *Athlete* has not been tested before, or requests an explanation of the procedure, the DCO shall explain the sample collection procedure to the *Athlete*.
- 5.7.9 As a minimum, the DCO shall ensure the *Athlete* is informed of his/her rights and responsibilities

5.8 *Selection of the sample collection vessel*

- 5.8.1 The *Athlete* shall be given a choice of sample collection vessels, from which they will be asked to choose one. It is recommended that there are at least 3 sample collection vessels from which to choose.
- 5.8.2 The *Athlete* and DCO shall check that the equipment is clean and intact. If either the *Athlete* or DCO is not satisfied with the equipment, the *Athlete* shall make another selection.
- 5.8.3 If the *Athlete* is not satisfied with any of the equipment, and the DCO does not agree with the *Athlete's* opinion that all of the available equipment is unsatisfactory, the DCO shall instruct the *Athlete* to proceed with the sample

collection session and the *Athlete's* views must be recorded on the doping control documentation by the DCO.

- 5.8.4 If both the DCO and the *Athlete* agree that none of the equipment is satisfactory, the DCO shall terminate sample collection, and record the reasons.
- 5.8.5 From this point, the sample collection vessel shall be handled only by the *Athlete* unless the *Athlete* authorizes the DCO/Chaperone or the *Athlete* Representative to handle the vessel on his/her behalf. Such authorization must be documented.

5.9 Sample Provision

- 5.9.1 The Witness (DCO or Chaperone) shall escort the *Athlete* to the toilet facility. The *Athlete* will carry his/her own sample collection vessel.
- 5.9.2 The Witness shall be of the same gender as the *Athlete* providing the sample.
- 5.9.3 The *Athlete* shall be encouraged to wash his/her hands before providing a sample.
- 5.9.4 Once in the toilet facility the *Athlete* must remove all clothing between the waist and mid-thigh, in order that the Witness has an unobstructed view of sample provision. Sleeves should be rolled up so that the *Athlete's* arms and hands are also clearly visible.
- 5.9.5 The Witness shall directly observe the *Athlete* provide the urine sample, adjusting his/her position so as to have a clear view of the sample leaving the *Athlete's* body.
- 5.9.6 The volume of urine collected should be that specified by the relevant laboratory.

NOTE: As a guideline, it is suggested that between 75ml and 100ml of urine be collected, depending on laboratory requirements. For EPO analysis, the recommended minimum is 100ml. However, the *Athlete* should be encouraged to fill the collection vessel.

- 5.9.7 Once a urine sample which satisfies the volume requirements has been collected, or the *Athlete* has provided a partial sample and is unable to provide any more urine at this time, the Witness shall escort the *Athlete*, who shall carry his/her own sample, back to the administration area.
- 5.9.8 If an *Athlete* wishes to wash his/her hands after passing the sample, the sample should at this time be placed in a safe and secure location, in full view of both the *Athlete* and the Witness.

- 5.9.9 If the Witness observes any unusual behavior by the *Athlete* while witnessing the passing of the sample, this should be reported to the Lead DCO as soon as possible, and documented.
- 5.9.10 The Witness shall sign the relevant documentation to verify that he/she witnessed sample provision in accordance with procedures

5.10. Insufficient Volume

- 5.10.1 In the event that an *Athlete* is unable to provide the required volume of urine, the DCO shall follow the procedure for a partial sample.
- 5.10.2 The DCO shall advise the *Athlete* that the partial sample provided shall be secured and a further sample collected.
- 5.10.3 The DCO shall instruct the *Athlete* to select partial sample equipment, as per 5.8. It is recommended that there are at least 3 partial sample kits from which to choose.
- 5.10.4 The DCO shall then instruct the *Athlete* to open the relevant equipment, pour the insufficient sample into the container, and seal it as directed by the DCO. The DCO shall check, in full view of the *Athlete*, that the container has been properly sealed.
- 5.10.5 The DCO and the *Athlete* shall check that the equipment code number and the volume and identity of the insufficient sample are recorded accurately by the DCO. The *Athlete* and DCO may initial or sign the documentation to show they are satisfied with the procedure.
- 5.10.6 The *Athlete* shall return to the waiting area, and remain under observation until ready to provide a further sample.
- 5.10.7 Either the *Athlete* or the DCO shall retain control of the sample. The DCO shall ensure that the sealed partial sample is securely stored (under continuous observation or locked away in a secure area). If the *athlete* retains possession of the sample, it must be placed in a secure area, and must remain under the observation of *sample collection personnel*.
- 5.10.8 When the *Athlete* is ready to provide more urine, the sample provision process shall recommence.
- 5.10.9 To ensure continuity of the process, and for the comfort of the *Athlete*, the Witness shall be the same Witness as for the initial attempt, whenever possible. However, a change of Witness shall in no way affect the integrity of the process.
- 5.10.10 The *Athlete* shall select a new sample collection vessel, and repeat the process as per 5.8.

- 5.10.11 This process shall be repeated until the DCO is satisfied that the *Athlete* has provided the required volume of urine once the initial and additional samples are combined.
- 5.10.12 The DCO shall ask the *Athlete* to inspect their partial sample(s) to ensure that the seals are secure. Any irregularities shall be recorded by the DCO on the doping control documentation or in a separate report to the ADO.
- 5.10.13 The DCO shall then direct the *Athlete* to break the seal of the partial sample container(s) and combine the samples in a new collection vessel, beginning with the first partial sample provided and each subsequent partial sample until the desired volume is reached.
- 5.10.14 Once the required volume of urine has been collected, the DCO and *Athlete* shall proceed to the next stage.

5.11 Dividing and sealing the sample

- 5.11.1 The *Athlete* shall select, from a choice of urine kits, a kit consisting of A and B containers, in which the sample is to be sealed. It is recommended that there are at least 3 urine kits from which to choose.
- 5.11.2 The *Athlete* and DCO shall check that the urine kit is clean and intact. If neither the *Athlete* nor the DCO is satisfied with the urine kit(s), the *Athlete* shall make another selection.
- 5.11.3 If the *Athlete* is not satisfied with any of the urine kits, and the DCO does not agree with the *Athlete's* opinion that all of the available urine kits are unsatisfactory, the DCO shall instruct the *Athlete* to proceed with the sample collection session, and the *Athlete's* views must be recorded on the doping control documentation by the DCO.
- 5.11.4 If both the DCO and the *Athlete* agree that none of the urine kits are satisfactory, the DCO shall terminate the session, and record the reasons.
- 5.11.5 The *Athlete* and the DCO shall check the urine kit to ensure that all the numbers of the A and B containers correspond.
- 5.11.6 If the numbers do not correspond, the DCO shall instruct the *Athlete* to select another kit. The DCO shall document this.
- 5.11.7 The DCO shall record the urine kit numbers and the *Athlete* and the DCO shall check the documentation to ensure that the DCO has accurately recorded the numbers of the A and B containers.
- 5.11.8 The *Athlete* shall pour the required minimum volume of urine into the B container. The recommended amount is 40% of the total.

- 5.11.9 The *Athlete* shall pour the remainder of the urine into the A container. The recommended amount is 60% of the total. The *Athlete* shall then pour any remaining urine into the B container, always leaving a residual amount of urine in the collection vessel.
- 5.11.10 The DCO shall instruct the *Athlete* in the sealing of the A and B containers. Both the DCO and the *Athlete* shall check that the bottles are securely sealed.
- 5.11.11 The DCO shall confirm that the sample meets the requirements for analysis, as specified by the ADO in accordance with the laboratory standards, by testing the residual volume of urine remaining in the collection vessel for specific gravity (greater than or equal to 1.005 if using a refractometer, or 1.010 with lab sticks, or as specified by the relevant laboratory) and, if necessary, pH (between 5 and 7, or as specified by the relevant laboratory). Reagent strips and/or a refractometer may be used.
- 5.11.12 The DCO shall ensure that any residual urine that will not be sent for analysis is discarded in full view of the *Athlete*.

5.12 Samples not meeting laboratory guidelines for analysis

- 5.12.1 If the reading is outside the required range for either specific gravity or, if required, pH, (5.11.11) the DCO shall request collection of additional sample(s), if so required by the ADO.
- 5.12.2 The ADO is responsible for establishing criteria for the number of additional samples to be collected at the session. If the additional samples collected do not meet the relevant laboratory's guidelines for analysis, the ADO is responsible for scheduling a new sample collection session for the *Athlete* and, if required, taking subsequent appropriate action.
- 5.12.3 While waiting to provide an additional sample the *Athlete* shall remain under continuous observation by a DCO/Chaperone.
- 5.12.4 When the *Athlete* is able to provide an additional sample, the DCO shall repeat the procedures for collection of the sample (5.8 to 5.11).
- 5.12.5 Whenever possible, provision of additional samples shall be observed by the same Witness as for the first. However, a change of Witness will not invalidate the sample collection procedure.
- 5.12.6 The Witness shall sign the relevant documentation to verify that he/she witnessed sample provision in accordance with procedures.
- 5.12.7 The DCO shall ensure that samples provided by the same *Athlete* can be linked through the documentation, and that the laboratory is informed which is the

initial sample. All samples shall be sent to the laboratory for analysis with all related paperwork.

5.13 Paperwork

NOTE: see WADA's standardized doping control documentation for an example of suitable documentation. See also the International Standard for Testing, 7.4.5. for minimum requirements.

- 5.13.1 If the *Athlete* provided more than one sample and the Witness was not the same individual that witnessed provision of the first sample, all Witnesses shall sign the doping control form.
- 5.13.2 If the Witness is unable to verify that he/she observed the passing of the sample, or reports unusual behavior by the *Athlete*, the Lead DCO can require the *Athlete* to provide a further sample. This must be documented, and all samples collected sent to the laboratory for analysis.
- 5.13.3 The DCO shall request the *Athlete* to provide information on all medications and/or supplements taken within the time period specified on the doping control form.

NOTE: The recommended period for medication information is 7 days

- 5.13.4 The DCO shall check all information on the form, fill in any incomplete areas in view of the *Athlete*, and sign to confirm that sample collection was conducted in accordance with procedures.
- 5.13.5 The *Athlete* and the Athlete's Representative, if present, shall be invited to check that all information on the form accurately reflects the details of the sample collection session. The *Athlete* shall be invited to complete the comments section of the form if he/she has any concerns or comments regarding the procedure. If there is insufficient space on the form, the *Athlete* shall be invited to complete a supplementary report form.
- 5.13.6 The Athlete's Representative, if present shall sign the Doping Control Form.
- 5.13.7 The *Athlete* and DCO shall then sign the Doping Control Form.
- 5.13.8 The DCO must give a full copy of the form to the *Athlete*.
- 5.13.9 Unless also required to provide a blood sample, the *Athlete* is then free to leave the *Doping Control Station*.

NOTE: If an Athlete is also required to provide a blood sample, and the doping control form records both blood and urine collection, the paperwork will not be fully completed until after collection of both blood and urine samples.

5.14 Sample Storage

- 5.14.1 The Lead DCO has the responsibility for ensuring, in accordance with the ADO criteria for sample storage, that all samples are stored in a manner that protects their identity, integrity and security whilst in the *Doping Control Station*.
- 5.14.2 Samples must not be left unattended, unless they are locked away in a refrigerator or cupboard, for example. Access shall be restricted to authorized personnel.
- 5.14.3 Where possible, samples shall be stored in a cool environment. Warm conditions should be avoided.
- 5.14.4 The DCO shall accurately complete appropriate documentation for each transport bag/container to ensure that the laboratory can verify the contents of the bag/container.
- 5.14.5 The DCO shall follow the ADO's system to ensure that, where required, instructions for the type of analysis to be conducted are provided to the laboratory.
- 5.14.6 The DCO shall complete the laboratory advice form/chain of custody form. The laboratory copy of this form(s) and the laboratory copy of the doping control form shall be placed in the transport bag with the samples, and sealed, preferably in the presence of a second person. Documentation identifying the *Athlete* shall not be included with the samples.
- 5.14.7 If relevant, the DCO shall record the times the transport bag is opened and re-sealed on the laboratory advice form or chain of custody form.
- 5.14.8 The DCO shall keep the samples secured and under his or her control until they are passed to the courier.
- 5.14.9 All documentation relevant to the testing session shall be forwarded to the ADO by the approved method as soon as possible after sample collection.

5.15 Transport of Samples

- 5.15.1 Samples shall be shipped to the WADA accredited laboratory as soon as practical, and wherever possible on the day of collection.
- 5.15.2 Samples may be taken directly to the laboratory by the DCO, or handed over to a third party for transportation. This third party must document the chain of custody of the samples. If an approved courier company is used to transport the samples, the DCO shall record the waybill number.

5.16 Hand-over of Samples to the laboratory

- 5.16.1 Laboratories are required to document receipt and the subsequent chain of custody of samples. Samples are reviewed for evidence of tampering or damage, and stored in appropriate conditions in accordance with the International Standard for Laboratories.

6 Modifications for Minors and Athletes with a Disability

Minors, or *Athletes* with specific types of disability may require modifications to the sample collection procedure. The modifications outlined below do not affect the integrity of the sample collection process.

The ADO has responsibility for ensuring, when possible, that the Lead DCO has any information and specialised sample collection equipment necessary to conduct a sample collection session with an *Athlete* with a disability requiring assistance during sample provision. The DCO shall have the authority to make modifications as the situation requires, in accordance with these guidelines.

In some cases, with the agreement of the Lead DCO, the *Athlete* may designate the Athlete Representative, or the DCO/Chaperone to assist with the sample collection process.

Any modifications made to the standard sample collection procedure shall be documented by the Lead DCO.

Modifications may be introduced in the case of the following *Athletes*:

6.2 *Minors*

- 6.2.1 *Minors* may, at their request, be accompanied by an Athlete Representative at all times during the sample collection procedure, including in the toilet area. However, the representative shall not witness the passing of the sample, unless requested to do so by the *Athlete*. The objective is to ensure that the Witness is observing sample provision correctly
- 6.2.2 The Athlete Representative or the DCO shall explain the doping control documentation to the *Athlete*, if necessary.
- 6.2.3 *Minors* must be accompanied for the verification of procedure (signing of the doping control form), and the Athlete Representative shall sign in addition to the *Athlete*.

6.3 *Athletes with restricted mobility/ restricted manual dexterity*

- 6.3.1 *Athletes* may ask the Athlete Representative or the DCO/Chaperone to assist them with mobility, when handling equipment, splitting the sample, or completing paperwork.
- 6.3.2 *Athletes* with Cerebral Palsy and/or significant lack of co-ordination may use a larger collection vessel, if available.
- 6.4 *Athletes with visual impairment*
- 6.4.1 *Athletes* may be accompanied by an Athlete Representative at all times during the sample collection procedure, including in the toilet area. However, the representative shall not witness the passing of the sample. The objective is to ensure that the Witness is observing sample provision correctly.
- 6.4.2 The Athlete Representative or the DCO shall read the doping control documentation to the *Athlete*, if necessary.
- 6.4.3 Blind *Athletes* must be accompanied for the verification of procedure (signing of the Doping Control Form), and the Athlete Representative shall sign on behalf of or in addition to the *Athlete*.
- 6.5 *Athletes with an intellectual disability*
- 6.5.1 *Athletes* shall be accompanied by an Athlete Representative at all times during sample collection procedure, including in the toilet area. However, the representative shall not witness the passing of the sample. The objective is to ensure that the Witness is observing sample provision correctly.
- 6.5.2 The Athlete Representative or the DCO shall read and/or explain the Doping Control documentation to the *Athlete*, if necessary.
- 6.5.3 *Athletes* with a intellectual disability must be accompanied for the verification of procedure (signing of the Doping Control Form), and the Athlete Representative shall sign on behalf of or in addition to the *Athlete*.
- 6.6 *Athletes using condom drainage or indwelling catheter drainage*
- 6.6.1 *Athletes* shall remove, or supervise the removal of, the existing collection bag and drain the system so that a fresh sample can be obtained.
- 6.7 *Athletes who self-catheterize*
- 6.7.1 *Athletes* may use their own catheter to provide a sample (this catheter should be produced in tamper-evident wrapping), or use one provided at the *Doping Control Station*, if available.

Appendix 1: Chaperone Training Guidelines

A. Procedure for training notifying Chaperones

1. The notifying Chaperones shall meet the Lead DCO at the *Doping Control Station* prior to the start of the event to receive training, instruction, credentials and assignments, as well as to complete a confidentiality agreement.
2. The Lead DCO shall first ensure that the notifying Chaperones meet the relevant criteria as specified by the ADO and are fit for duty. If not, alternative Chaperones should be found.
3. The Lead DCO shall specifically ask the notifying Chaperones if they have any conflict of interest, such as involvement with any *Athlete* that might be tested. If so, alternative Chaperones should be found.
4. The Lead DCO shall ensure that all notifying Chaperones have a watch, and all staff should synchronize their watches.
5. The Lead DCO shall review or arrange for the review of the notification and escorting procedure with the notifying Chaperones. (5.5/5.6) The explanation should include the purpose of the Chaperone role, and what to do if problems are encountered. The DCO should make it clear that a Chaperone should ask for assistance from the Lead DCO if they are not sure how to proceed at any time.
6. The DCO shall demonstrate the notification procedure by means of role-play, should observe the Chaperone practice at least once, and point out mistakes as well as providing praise.
7. The DCO shall inform the Chaperones that any strange behavior by *Athletes*, such as ingestion of unknown substances, or evasion, should be discretely reported to the Lead DCO upon arrival at the *Doping Control Station*.
8. The DCO shall encourage questions from the Chaperones.
9. The Lead DCO shall arrange a location and time for the Chaperones to receive information specific to the *Athlete* they will be notifying.

NOTE: whenever possible, notifying Chaperones should be assigned to *Athletes* of the same gender.

10. Notifying Chaperones shall then sign the relevant agreement which should as a minimum require a commitment to confidentiality and outline a code of conduct. The form should also require the Notifying Chaperone to provide their contact details and date of birth.

11. The Chaperones shall, where possible, have an opportunity to assess the location and plan the approach and timing of notification, taking into account the specific circumstances of the session.
12. The Lead DCO shall provide guidelines as to the Chaperone's responsibilities after the *Athlete* has arrived at the *Doping Control Station* (i.e. whether they are free to leave, or have further responsibilities).

B Procedure for training witnessing Chaperones

Ideally, witnessing Chaperones will have prior experience, and will not be trained on-site.

1. The witnessing Chaperones shall meet the Lead DCO at the *Doping Control Station* prior to the start of the event to receive training, instruction, credentials and assignments, as well as to complete the confidentiality agreement. Training should be separate from that of the Notifying Chaperones.
2. The Lead DCO shall first ensure that the witnessing Chaperones meet the relevant criteria as specified by the ADO and are fit for duty. If not, alternative Chaperones should be found.
3. The Lead DCO shall specifically ask the Chaperones if they have any conflict of interest, such as involvement with any *Athlete* that might be tested. If not, alternative Chaperones should be found.
4. The Lead DCO shall review, or arrange the review of, the specific procedure for observation of sample provision (5.9).
5. Witnessing Chaperones shall also be briefed about any sensitivities, and any potential scenarios which might be encountered.
6. The DCO shall encourage questions from the Chaperones.
7. Witnessing Chaperones shall then sign the relevant agreement which should as a minimum require a commitment to confidentiality and outline a code of conduct. The form should also require the Witnessing Chaperone to provide their contact details and date of birth.
8. The Lead DCO shall provide guidelines as to the Chaperone's responsibilities after the *Athlete* has provided the Sample at the *Doping Control Station* (i.e. whether they are free to leave, or have further responsibilities).

WADA Technical Document – TD2003IDCR

| | | | |
|------------------|-------------------|-----------------|-----------------|
| Document Number: | TD2003IDCR | Version Number: | 1.2 |
| Written by: | WADA Project Team | Approved by: | |
| Date: | May 11, 2003 | Effective Date: | January 1, 2004 |

IDENTIFICATION CRITERIA FOR QUALITATIVE ASSAYS INCORPORATING CHROMATOGRAPHY AND MASS SPECTROMETRY

The appropriate analytical characteristics must be documented for a particular assay. The Laboratory must establish criteria for identification of a compound. Examples of acceptable criteria are:

Chromatographic separation

For capillary gas chromatography, the retention time (RT) of the analyte shall not differ by more than one (1) percent or ± 0.2 minutes (whichever is smaller) from that of the same substance in a spiked urine sample, Reference Collection sample, or Reference Material analyzed contemporaneously. In those cases where shifts in retention can be explained, for example by sample overload, the retention time criteria may be relaxed. For high performance liquid chromatography, the RT of the analyte shall not differ by more than two (2) percent or ± 0.4 minutes (whichever is smaller) from that of the same substance in a spiked urine sample, Reference Collection sample, or Reference Material analyzed in the same analytical batch.

Mass Spectrometric Detection

Full scan mode: A full or partial **scan** is the preferred approach to identification. A partial **scan** may begin at an m/z value greater than any abundant ion due to the derivatizing agent or chemical ionization reagent.

When a full or partial **scan** is acquired, all **diagnostic ions** with a **relative abundance** greater than 10% in the reference spectrum obtained from a positive control urine, a Reference Collection sample, or a Reference Material must be present in the spectrum of the unknown peak. In addition, the **relative abundance** of three **diagnostic ions** shall not differ by more than the amount shown in Table 1 from the relative intensities of the same ions from that of a spiked urine, a Reference Collection sample, or a Reference Material. The **relative abundance** of the **diagnostic ions** may be obtained from single or averaged spectra or integration of peak areas of extracted ion profiles.

Background subtraction, if applied, should be performed uniformly on all *Samples* analyzed contemporaneously and used to make decisions regarding the presence of a *Prohibited Substance* or *Method*, its *Metabolite*, or *Marker*.

The use of computer-based mass spectral library searching or matching is permitted. The laboratory must establish criteria for acceptance of compound identification based on spectral match quality. Since the match factor of a reverse search does not guarantee identification, all spectral library matches must be reviewed by a qualified scientist.

If three **diagnostic ions** with a **relative abundance** greater than 5% are not available, a second derivative shall be prepared, or a second ionization or fragmentation technique shall be used. The second derivative should yield different

WADA Technical Document – TD2003IDCR

| | | | |
|------------------|-------------------|-----------------|-----------------|
| Document Number: | TD2003IDCR | Version Number: | 1.2 |
| Written by: | WADA Project Team | Approved by: | |
| Date: | May 11, 2003 | Effective Date: | January 1, 2004 |

diagnostic ions. The second ionization technique must be based on a different physical principle, i.e., chemical ionization vs. electronic ionization and again should provide different diagnostic ions. It is not acceptable to utilize a technique that changes only the **relative abundance** of the same mass ions. In any case, a minimum of two **diagnostic ions** is mandatory in each mass spectrum.

Selected Ion Monitoring Mode: In some cases, it may be necessary to monitor selected ions in order to detect the substance at the Minimum Required Performance Limits. When selected ions are monitored, at least three **diagnostic ions** must be acquired. The **relative abundance** of a **diagnostic ion** shall preferably be determined from the peak area or height of integrated selected ion chromatograms. The **signal-to-noise ratio** of the least intense **diagnostic ion** must be greater than three to one (3:1). The relative intensities of any of the ions shall not differ by more than the amount in Table 1 from the relative intensities of the same ions acquired from a spiked urine, Reference Collection sample, or Reference Material. For a **diagnostic ion** with a **relative abundance** of less than 5% in the reference, the ion must be present in the unknown. The concentration of *Prohibited Substance*, or its *Metabolite*, or its *Marker* should be comparable in the *Sample* and the spiked urine, Reference Collection sample, or Reference Material.

Table 1
Maximum Tolerance Windows for Relative Ion Intensities
to Ensure Appropriate Uncertainty in Identification

| Relative Abundance (% of base peak) | EI-GC/MS | CI-GC/MS; GC/MS ⁿ ; LC/MS ; LC/MS ⁿ |
|--|------------------|---|
| > 50% | ±10% (absolute) | ± 15% (absolute) |
| 25% to 50% | ± 20% (relative) | ±25% (relative) |
| < 25% | ±5% (absolute) | ±10% (absolute) |

If the Laboratory protocol requires three ions to be within a tolerance window to identify a substance, it is not permissible to collect additional ions and select those ion ratios that are within tolerance and ignore others that would not result in meeting identification criteria without a valid explanation.

If three **diagnostic ions** are not available, a second derivative shall be prepared, or a second ionization or fragmentation technique shall be used. The second derivative should yield different **diagnostic ions**. The second ionization technique must be based on a different physical principle, i.e., chemical ionization vs. electronic ionization and again should provide different **diagnostic ions**. It is not acceptable to utilize a technique that changes only the **relative abundance** of the

WADA Technical Document – TD2003IDCR

| | | | |
|------------------|-------------------|-----------------|-----------------|
| Document Number: | TD2003IDCR | Version Number: | 1.2 |
| Written by: | WADA Project Team | Approved by: | |
| Date: | May 11, 2003 | Effective Date: | January 1, 2004 |

Definitions

Diagnostic ion(s): Molecular ion or fragment ions whose presence and abundance are characteristic of the substance and thereby may assist in its identification. A second ion belonging to the same isotopic cluster may also be used as diagnostic only when the peculiarity of the atomic composition of the fragment so justifies (e.g. presence of Cl, Br, or other elements with abundant isotopic ions).

High resolution mass spectrometry (HRMS): For the purposes of the *International Standards for Laboratories*, HRMS is defined as mass spectrometry at a resolving power (10 % valley definition) in excess of 3,000.

Low resolution mass spectrometry (LRMS): LRMS is defined as mass spectrometry at a resolving power (10 % valley definition) lower than 3,000.

Relative abundance (mass spectrometry): The abundance of a particular ion relative to the most abundant ion monitored expressed as a percentage.

Maximum difference in relative abundance: The maximum permitted difference between the relative abundance of a particular ion obtained from the *Sample* and that obtained from the positive control urine. This may be expressed in ABSOLUTE or RELATIVE terms.

Absolute difference: Calculated by subtracting the stated percentage from the relative abundance obtained for the studied ion from the positive control urine or Reference Material. For example, if the relative abundance of an ion in the chromatographic peak of interest in the positive control urine or Reference Material is measured as 20%, then the observed relative abundance for the same ion in the peak of interest in the unknown urine sample would be required to be in the range of 15-25% ($20\% \pm 5\%$) for the ion to contribute to an acceptable identification.

Relative difference: Calculated by multiplying the stated percentage by the relative abundance obtained for the studied ion from the positive control urine or Reference Material. For example, if the relative abundance of an ion in the chromatographic peak of interest in the positive control urine or Reference Material appears as 30 % and the stated maximum permitted difference is 20 % (relative), then the observed relative abundance for the same ion in the peak of interest in the unknown urine sample would be required to be in the range of 24-36% ($30\% \pm (30 \times 20\%)$) for the ion to contribute to an acceptable identification.

Scan: Acquisition of ions of a continuous range of m/z values.

Selected ion monitoring (SIM): Acquisition of ions of one or more pre-determined discrete m/z values for specified dwell times.

WADA Technical Document – TD2003IDCR

| | | | |
|------------------|-------------------|-----------------|-----------------|
| Document Number: | TD2003IDCR | Version Number: | 1.2 |
| Written by: | WADA Project Team | Approved by: | |
| Date: | May 11, 2003 | Effective Date: | January 1, 2004 |

Signal-to-Noise Ratio: Magnitude of the instrument response to the analyte (signal) relative to the magnitude of the background (noise).

Tandem mass spectrometry (MS/MS or MSⁿ): A technique in which a precursor ion is isolated in a mass analyzer, fragmented by collision with a collision gas, and the product ions collected in a second mass analyzer. This process can be applied multiple times, each application being reflected in the "n" exponent. The technique may be accomplished either in space (e.g. triple quadrupole MS) or in time (e.g. ion trap MS)

Data File : K:\CHEM\AAS\CONFIRM\MSDA13\9CS03LR.D

Acquired : 29 Jun 6:47 pm

Instrument : MSDA13

Method File: ANAB97LS

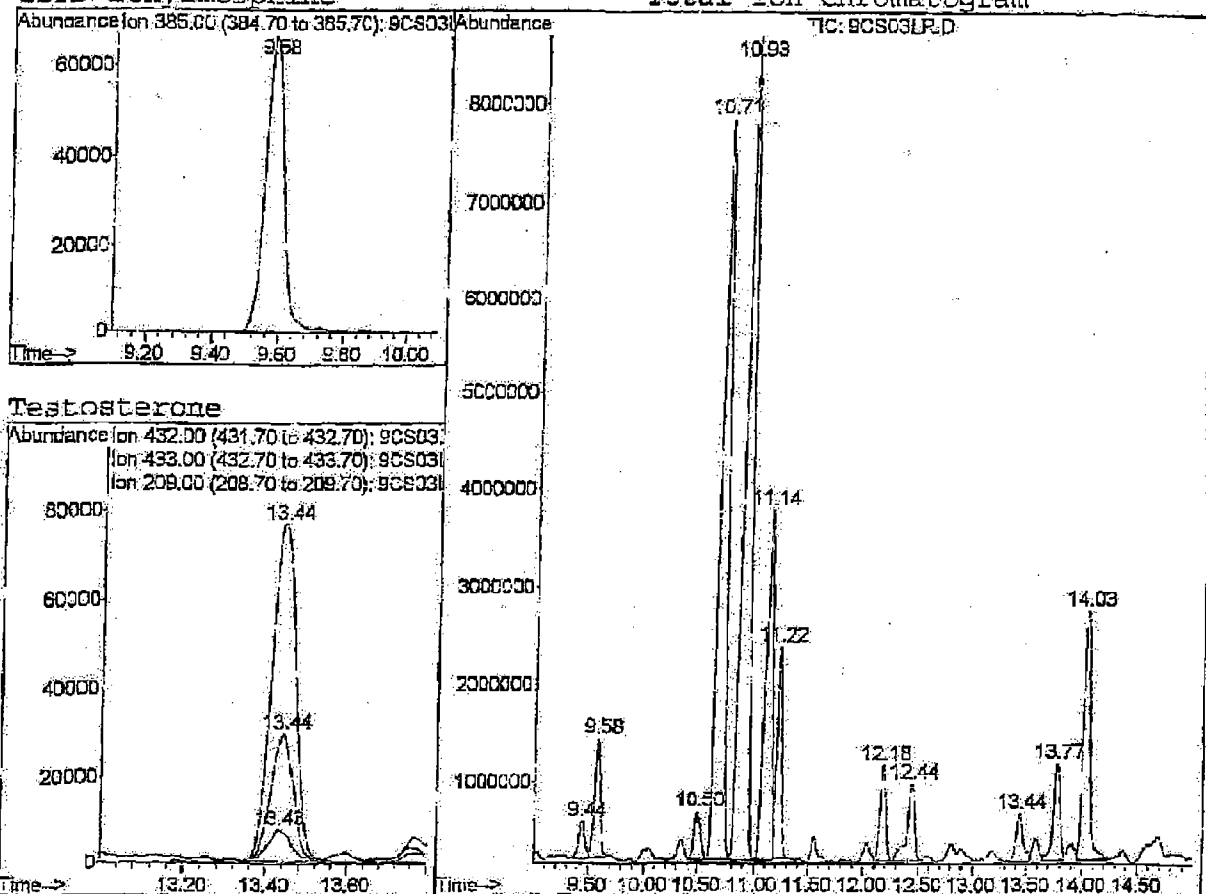
Misc Info :

Vial Number: 84

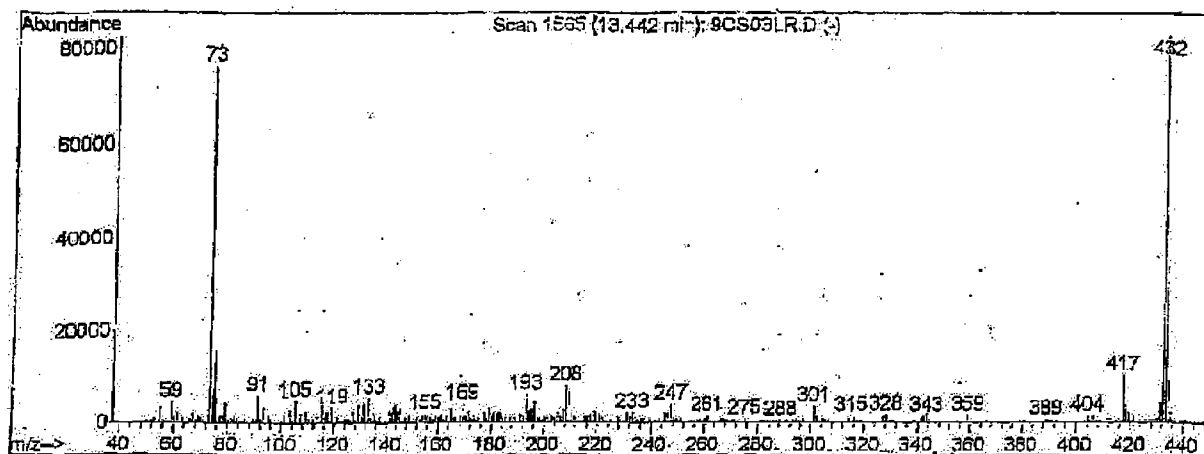
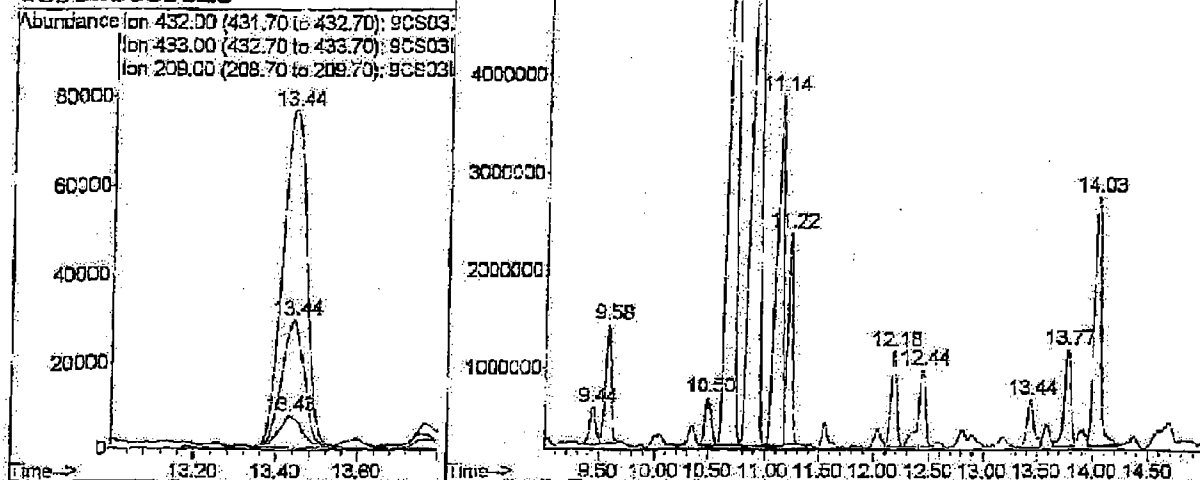
Sample Name: 9CS03 TE A CONFIRMATION LIN

ISTD Ethylmorphine

Total Ion Chromatogram



Testosterone



Handwritten signature/initials

GC/C/IRMS - Results

26.02.03

Federation: [REDACTED]

Event: [REDACTED]

Code-Nr.: 188702

Lab-Nr.: 1013/03 A

Sample receipt: 11.02.03

Results: The ratio testosterone/epitestosterone is higher than 6
(25.3 ± 0.31, n=3); the GC/C/IRMS results indicate an application
of testosterone or testosterone prohormones

For the sample with the code number 188702 the following $\delta^{13}\text{C}$ [‰] - values were obtained:

testosterone metabolites:

etiocholanolone - 30.5 ± 0.2 (n=5)

androsterone - 29.5 ± 0.3 (n=5)

internal reference compounds:

11 β -hydroxy-androsterone - 20.46; - 20.17

pregnanediol - 21.1 ± 0.1 (n=3)

Conclusions

The $\delta^{13}\text{C}$ [‰]-values of the testosterone metabolites indicate the application of testosterone
or testosterone prohormones.

DEUTSCHE SPORTHOCHSCHULE
KÖLN
INSTITUT FÜR BIOCHEMIE
IOC AKKREDITIERTES LABOR
FÜR DOPINGANALYTIK

Prof. Dr. Wilhelm Schänzer * Carl-Diem-Weg 6 * 50933 Köln

Prof. Dr. Wilhelm Schänzer
C/O Deutsche Sporthochschule Köln
INSTITUT FÜR BIOCHEMIE
Carl-Diem-Weg 6
50933 Köln

TELEFON: 0221-4971313
TELEFAX: 0221-4973236

total pages -1-

KÖLN, DEN 8. April 2003

Documentation Analysis Report 202/03

Code: 188702

Decision criterias for GC/C/IRMS results

Based on our reference values the following criterias indicate an application of testosterone or testosterone prohormones:

Difference of the $\delta^{13}\text{C}$ [‰]- values between androsterone and pregnanediol > 2.6 or
difference between the $\delta^{13}\text{C}$ [‰]- values of etiocholanolone and pregnanediol > 3.3


Prof. Dr. Wilhelm Schänzer

| | | | | |
|----|-------|--------|-----|-----|
| | NR | 05/751 | PR | SG |
| S1 | IN | 8/4 | TES | MED |
| S2 | OUT | | IN | SG |
| S3 | INDEX | | REG | AP |

GDC0403

THE LIST

THE 2005 PROHIBITED LIST
INTERNATIONAL STANDARD

WADA
play true

THE 2005 PROHIBITED LIST INTERNATIONAL STANDARD

THE WORLD ANTI-DOPING CODE

2005 Prohibited List

Published by:

World Anti-Doping Agency
www.wada-ama.org
info@wada-ama.org

Headquarters

800 Place Victoria - Suite 1700
P.O. Box 120
Montreal, QC
Canada H4Z 1B7
Tel.: +1.514.904.9232
Fax: +1.514.904.8650

African Regional Office

Protea Assurance Building
8th Floor
Greenmarket Square
Cape Town
8001 South Africa
Tel.: +27.21.483.9790
Fax: +27.21.483.9791

Asia/Oceania Regional Office

C/O Japan Institute of Sports Sciences
3-15-1 Nishigaoka, Kita-Ku
Tokyo
115-0056 Japan
Tel.: +81.3.5963.4321
Fax: +81.3.5963.4320

European Regional Office

Avenue du Tribunal-Fédéral 34
1005 Lausanne
Switzerland
Tel.: +41.21.343.43.40
Fax: +41.21.343.43.41

The official text of the *Prohibited List* shall be maintained by WADA and shall be published in English and French. In the event of any conflict between the English and French versions, the English version shall prevail.

This List is effective as of 1 January 2005.

The use of any drug should be limited to medically justified indications.



TABLE OF CONTENTS

SUBSTANCES AND METHODS PROHIBITED AT ALL TIMES (IN- AND OUT-OF-COMPETITION)

PROHIBITED SUBSTANCES

| | |
|--|----|
| S1. ANABOLIC AGENTS | 05 |
| S2. HORMONES AND RELATED SUBSTANCES | 08 |
| S3. BETA-2 AGONISTS | 08 |
| S4. AGENTS WITH ANTI-ESTROGENIC ACTIVITY | 09 |
| S5. DIURETICS AND OTHER MASKING AGENTS | 10 |

PROHIBITED METHODS

| | |
|--|----|
| M1. ENHANCEMENT OF OXYGEN TRANSFER | 11 |
| M2. CHEMICAL AND PHYSICAL MANIPULATION | 11 |
| M3. GENE DOPING | 11 |

SUBSTANCES AND METHODS PROHIBITED IN-COMPETITION

INCLUDES S1 TO S5 AND M1 TO M3 DEFINED ABOVE, AS WELL AS:

| | |
|--------------------------------|----|
| S6. STIMULANTS | 13 |
| S7. NARCOTICS | 14 |
| S8. CANNABINOIDS | 14 |
| S9. GLUCOCORTICOSTEROIDS | 14 |

SUBSTANCES PROHIBITED IN PARTICULAR SPORTS

| | |
|-------------------------|----|
| P1. ALCOHOL | 17 |
| P2. BETA-BLOCKERS | 18 |

| | |
|----------------------------|----|
| SPECIFIED SUBSTANCES | 19 |
|----------------------------|----|

THE WORLD ANTI-DOPING CODE

SUBSTANCES & METHODS PROHIBITED AT ALL TIMES

IN- AND OUT-OF-COMPETITION

PROHIBITED SUBSTANCES



S1. ANABOLIC AGENTS

Anabolic agents are prohibited.

1. Anabolic Androgenic Steroids (AAS)

a. Exogenous* AAS, including:

| | |
|--|-----------------------|
| 18 α -homo-17 β -hydroxyestr- 4-en-3-one | metenolone |
| bolasterone | methandienone |
| boldenone | methandriol |
| boldione | methyldienolone |
| calusterone | methyltrienolone |
| clostebol | methyltestosterone |
| danazol | mibolerone |
| dehydrochloromethyl- testosterone | nandrolone |
| delta1-androstene-3,17-dione | 19-norandrostenediol |
| delta1-androstenediol | 19-norandrostenedione |
| delta1-dihydro-testosterone | norbolethone |
| drostanolone | norclostebol |
| ethylestrenol | norethandrolone |
| fluoxymesterone | oxabolone |
| formebolone | oxandrolone |
| furazabol | oxymesterone |
| gestrinone | oxymetholone |
| 4-hydroxytestosterone | quinbolone |
| 4-hydroxy-19-nortestosterone | stanozolol |
| mestanolone | stenbolone |
| mesterolone | tetrahydrogestrinone |
| | trenbolone |

and other substances with a similar chemical structure or similar biological effect(s).

*"exogenous" refers to a substance which is not capable of being produced by the body naturally.

THE 2005 PROHIBITED LIST

05

b. Endogenous** AAS:

androstenediol (androst-5-ene-3 β ,17 β -diol)
androstenedione (androst-4-ene-3,17-dione)
dehydroepiandrosterone (DHEA)
dihydrotestosterone
testosterone

and the following metabolites and isomers:

5 α -androstane-3 α ,17 α -diol
5 α -androstane-3 α ,17 β -diol
5 α -androstane-3 β ,17 α -diol
5 α -androstane-3 β ,17 β -diol
androst-4-ene-3 α ,17 α -diol
androst-4-ene-3 α ,17 β -diol
androst-4-ene-3 β ,17 α -diol
androst-5-ene-3 α ,17 α -diol
androst-5-ene-3 α ,17 β -diol
androst-5-ene-3 β ,17 α -diol
4-androstenediol (androst-4-ene-3 β ,17 β -diol)
5-androstenedione (androst-5-ene-3,17-dione)
epi-dihydrotestosterone
3 α -hydroxy-5 α -androstan-17-one
3 β -hydroxy-5 α -androstan-17-one
19-norandrosterone
19-noretiocholanolone

Where a *Prohibited Substance* (as listed above) is capable of being produced by the body naturally, a *Sample* will be deemed to contain such *Prohibited Substance* where the concentration of the *Prohibited Substance* or its metabolites or markers and/or any other relevant ratio(s) in the *Athlete's Sample* so deviates from the range of values normally found in humans that it is unlikely to be consistent with normal endogenous production. A *Sample* shall not be deemed to contain a *Prohibited Substance* in any such case where the *Athlete* proves by evidence that the concentration

** "endogenous" refers to a substance which is capable of being produced by the body naturally.

of the *Prohibited Substance* or its metabolites or markers and/or the relevant ratio(s) in the *Athlete's Sample* is attributable to a physiological or pathological condition. In all cases, and at any concentration, the laboratory will report an *Adverse Analytical Finding* if, based on any reliable analytical method, it can show that the *Prohibited Substance* is of exogenous origin.

If the laboratory result is not conclusive and no concentration as referred to in the above paragraph is found, the relevant *Anti-Doping Organization* shall conduct a further investigation if there are serious indications, such as a comparison to reference steroid profiles, for a possible *Use of a Prohibited Substance*.

If the laboratory has reported the presence of a T/E ratio greater than four (4) to one (1) in the urine, further investigation is obligatory in order to determine whether the ratio is due to a physiological or pathological condition, except if the laboratory reports an *Adverse Analytical Finding* based on any reliable analytical method, showing that the *Prohibited Substance* is of exogenous origin.

In case of an investigation, it will include a review of any previous and/or subsequent tests. If previous tests are not available, the *Athlete* shall be tested unannounced at least three times within a three month period.

Should an *Athlete* fail to cooperate in the investigations, the *Athlete's Sample* shall be deemed to contain a *Prohibited Substance*.

2. Other Anabolic Agents, including but not limited to:

clenbuterol
zeranol
zilepaterol

S2. HORMONES AND RELATED SUBSTANCES

The following substances, including other substances with a similar chemical structure or similar biological effect(s), and their releasing factors, are prohibited.

1. Erythropoietin (EPO)
2. Growth Hormone (hGH), Insulin-like Growth Factor (IGF-1), Mechano Growth Factors (MGFs)
3. Gonadotrophins (LH, hCG)
4. Insulin
5. Corticotrophins

Unless the *Athlete* can demonstrate that the concentration was due to a physiological or pathological condition, a *Sample* will be deemed to contain a *Prohibited Substance* (as listed above) where the concentration of the *Prohibited Substance* or its metabolites and/or relevant ratios or markers in the *Athlete's Sample* so exceeds the range of values normally found in humans so that it is unlikely to be consistent with normal endogenous production.

The presence of other substances with a similar chemical structure or similar biological effect(s), diagnostic marker(s) or releasing factors of a hormone listed above or of any other finding which indicate(s) that the substance detected is of exogenous origin, will be reported as an *Adverse Analytical Finding*.

S3. BETA-2 AGONISTS

All beta-2 agonists including their D- and L-isomers are prohibited. Their use requires a Therapeutic Use Exemption.

As an exception, formoterol, salbutamol, salmeterol and terbutaline, when administered by inhalation to prevent and/or treat asthma and exercise-induced asthma/broncho-constriction require an abbreviated Therapeutic Use Exemption.

Despite the granting of a Therapeutic Use Exemption, when the Laboratory has reported a concentration of salbutamol (free plus glucuronide) greater than 1000 ng/mL, this will be considered as an *Adverse Analytical Finding* unless the athlete proves that the abnormal result was the consequence of the therapeutic use of inhaled salbutamol.

S4. AGENTS WITH ANTI-ESTROGENIC ACTIVITY

The following classes of anti-estrogenic substances are prohibited:

1. Aromatase inhibitors including, but not limited to:

| | |
|-------------------|--------------|
| anastrozole | formestane |
| aminoglutethimide | letrozole |
| exemestane | testolactone |

2. Selective Estrogen Receptor Modulators (SERMs) including, but not limited to:

raloxifene
tamoxifen
toremifene

3. Other anti-estrogenic substances including, but not limited to:

clomiphene
cyclofenil
fulvestrant

S5. DIURETICS AND OTHER MASKING AGENTS

Diuretics and other masking agents are prohibited.
Masking agents include but are not limited to:

diuretics*
epitestosterone
probenecid
alpha-reductase inhibitors
(e.g. finasteride, dutasteride)
plasma expanders
(e.g. albumin, dextran, hydroxyethyl starch)

Diuretics include:

| | |
|----------------|--|
| acetazolamide | indapamide |
| amiloride | metolazone |
| bumetanide | spironolactone |
| canrenone | thiazides (e.g. bendroflumethiazide, chlorothiazide, hydrochlorothiazide) |
| chlortalidone | triamterene |
| etacrynic acid | |
| furosemide | |

and other substances with a similar chemical structure or similar biological effect(s).

* A Therapeutic Use Exemption is not valid if an Athlete's urine contains a diuretic in association with threshold or sub-threshold levels of a Prohibited Substance(s).

PROHIBITED METHODS



M1. ENHANCEMENT OF OXYGEN TRANSFER

The following are prohibited:

- a. Blood doping, including the use of autologous, homologous or heterologous blood or red blood cell products of any origin, other than for medical treatment.
- b. Artificially enhancing the uptake, transport or delivery of oxygen, including but not limited to perfluorochemicals, efaproxiral (RSR13) and modified haemoglobin products (e.g. haemoglobin-based blood substitutes, microencapsulated haemoglobin products).

M2. CHEMICAL AND PHYSICAL MANIPULATION

The following is prohibited:

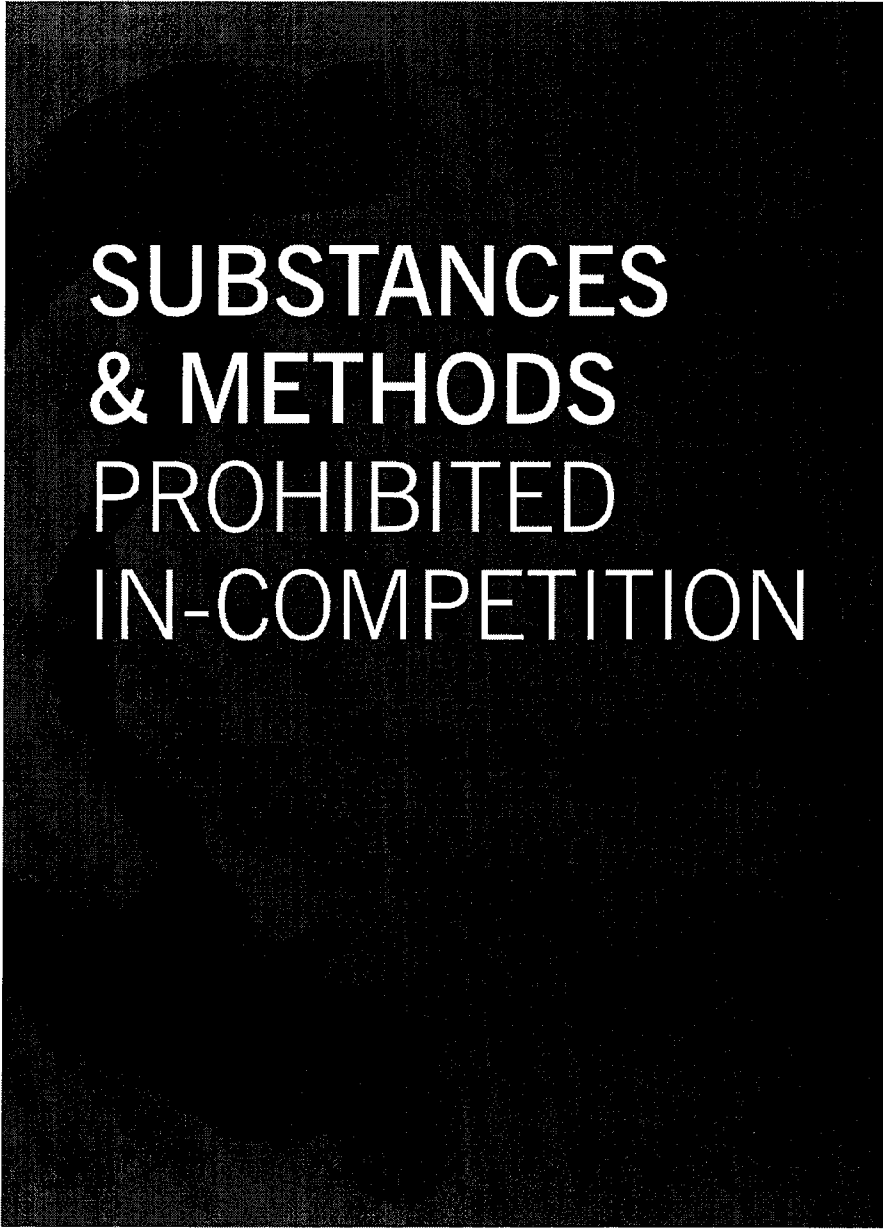
Tampering, or attempting to tamper, in order to alter the integrity and validity of *Samples* collected in *Doping Controls*.

These include but are not limited to intravenous infusions*, catheterization, and urine substitution.

M3. GENE DOPING

The non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance, is prohibited.

* Except as a legitimate acute medical treatment, intravenous infusions are prohibited.

A dark, textured rectangular area, possibly a book cover or a graphic element, with white text centered on it. The text is in a bold, sans-serif font and is arranged in four lines.

SUBSTANCES & METHODS PROHIBITED IN-COMPETITION

In addition to the previously listed categories S1, S2, S3, S4, S5 and M1, M2, M3, the following are prohibited *in-competition*:



S6. STIMULANTS

The following stimulants are prohibited, including both their optical (D- and L-) isomers where relevant:

| | |
|---------------------|-------------------------------|
| adrafinil | furfenorex |
| amfepramone | mefenorex |
| amiphenazole | mephentermine |
| amphetamine | mesocarb |
| amphetaminil | methamphetamine |
| benzphetamine | methylamphetamine |
| bromantan | methylenedioxymphetamine |
| carphedon | methylenedioxymethamphetamine |
| cathine* | methylephedrine** |
| clobenzorex | methylphenidate |
| cocaine | modafinil |
| dimethylamphetamine | nikethamide |
| ephedrine** | norfenfluramine |
| etilamphetamine | parahydroxyamphetamine |
| etilefrine | pemoline |
| famprofazone | phendimetrazine |
| fencamfamin | phenmetrazine |
| fencamine | phentermine |
| fenetylline | prolintane |
| fenfluramine | selegiline |
| fenproporex | strychnine |

and other substances with a similar chemical structure or similar biological effect(s)***.

NOTE: Adrenaline associated with local anaesthetic agents or by local administration (e.g. nasal, ophthalmologic) is not prohibited.

* Cathine is prohibited when its concentration in urine is greater than 5 micrograms per milliliter.

** Each of ephedrine and methylephedrine is prohibited when its concentration in urine is greater than 10 micrograms per milliliter.

*** The substances included in the 2005 Monitoring Program (bupropion, caffeine, phenylephrine, phenylpropanolamine, pipradrol, pseudoephedrine, synephrine) are not considered as Prohibited Substances.

S7. NARCOTICS

The following narcotics are prohibited:

| | |
|------------------------------|-------------|
| buprenorphine | morphine |
| dextromoramide | oxycodone |
| diamorphine (heroin) | oxymorphone |
| fentanyl and its derivatives | pentazocine |
| hydromorphone | pethidine |
| methadone | |

S8. CANNABINOIDS

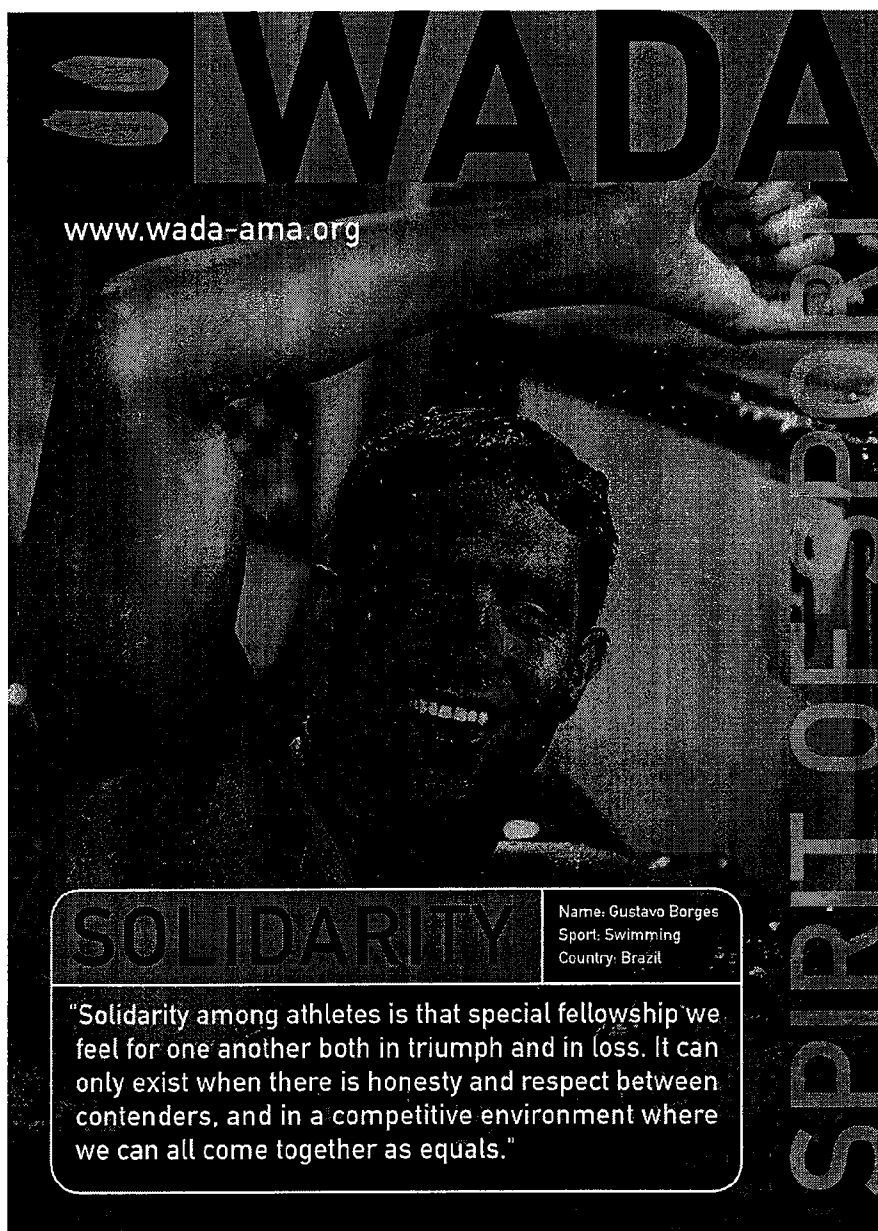
Cannabinoids (e.g. hashish, marijuana) are prohibited.

S9. GLUCOCORTICOSTEROIDS

All glucocorticosteroids are prohibited when administered orally, rectally, intravenously or intramuscularly. Their use requires a Therapeutic Use Exemption approval.

All other routes of administration require an abbreviated Therapeutic Use Exemption.

Dermatological preparations are not prohibited.



WADA

www.wada-ama.org

SOLIDARITY

Name: Gustavo Borges
Sport: Swimming
Country: Brazil

"Solidarity among athletes is that special fellowship we feel for one another both in triumph and in loss. It can only exist when there is honesty and respect between contenders, and in a competitive environment where we can all come together as equals."

SPIRIT OF SPORT



SUBSTANCES PROHIBITED IN PARTICULAR SPORTS

PROHIBITED SUBSTANCES



P1. ALCOHOL

Alcohol (ethanol) is prohibited *in-competition* only, in the following sports. Detection will be conducted by analysis of breath and/or blood. The doping violation threshold for each Federation is reported in parenthesis.

| | |
|--|------------|
| Aeronautic (FAI) | (0.20 g/L) |
| Archery (FITA) | (0.10 g/L) |
| Automobile (FIA) | (0.10 g/L) |
| Billiards (WCBS) | (0.20 g/L) |
| Boules (CMSB) | (0.10 g/L) |
| Karate (WKF) | (0.10 g/L) |
| Modern Pentathlon (UIPM) for disciplines involving shooting | (0.10 g/L) |
| Motorcycling (FIM) | (0.00 g/L) |
| Skiing (FIS) | (0.10 g/L) |

P2. BETA-BLOCKERS

Unless otherwise specified, beta-blockers are prohibited *in-competition* only, in the following sports:

Aeronautic (FAI)
 Archery (FITA) (also prohibited *out-of-competition*)
 Automobile (FIA)
 Billiards (WCBS)
 Bobsleigh (FIBT)
 Boules (CMSB)
 Bridge (FMB)
 Chess (FIDE)
 Curling (WCF)
 Gymnastics (FIG)
 Motorcycling (FIM)
 Modern Pentathlon (UIPM) for disciplines involving shooting
 Nine-pin bowling (FIQ)
 Sailing (ISAF) for match race helms only
 Shooting (ISSF) (also prohibited *out-of-competition*)
 Skiing (FIS) in ski jumping & free style snow board
 Swimming (FINA) in diving & synchronised swimming
 Wrestling (FILA)

Beta-blockers include, but are not limited to, the following:

| | | |
|------------|--------------|-------------|
| acebutolol | carvedilol | nadolol |
| alprenolol | celiprolol | oxprenolol |
| atenolol | esmolol | pindolol |
| betaxolol | labetalol | propranolol |
| bisoprolol | levobunolol | sotalol |
| bunolol | metipranolol | timolol |
| carteolol | metoprolol | |

SPECIFIED SUBSTANCES

"Specified Substances"* are listed below.

ephedrine
L-methylamphetamine
methylephedrine
cannabinoids
all inhaled beta-2 agonists, except clenbuterol
probenecid
all glucocorticosteroids
all beta blockers
alcohol

* "The Prohibited List may identify specified substances which are particularly susceptible to unintentional anti-doping rule violations because of their general availability in medicinal products or which are less likely to be successfully abused as doping agents. "A doping violation involving such substances may result in a reduced sanction provided that the Athlete can establish that the use of such a specified substance was not intended to enhance sport performance..."

ionisation modes, i.e. ESI and APCI, can be employed for the detection of steroid glucuronide conjugates, and main reasons for inconclusive results are based on chromatographic issues.

Acknowledgements

The scientific support of Dr. Michel Niesen is gratefully acknowledged by the project consortium as well as the cooperation with RIKIL, Institute of Food Safety and the doping control laboratories of Kneissle, London and Oslo.

References

1. Schänzer W, Donike M. Metabolism of androgenic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Anal. Chim. Acta* 1993; 275: 23.
2. Schänzer W. Metabolism of anabolic androgenic steroids. *Clin. Chem.* 1996; 42: 1601.
3. Schänzer W, Donike M. Synthesis of deuterated steroids for GC/MS quantification of endogenous steroids. In Donike M (Ed.), *Proceedings of the 12th Cologne Workshop in Dope Analysis*, 1994, Sport und Buch Strauß, Köln, 1995: 93.
4. Thevis M, Opfermann G, Schmickler H, Schänzer W. Mass spectrometry of steroid glucuronide conjugates. I. Electron impact fragmentation of 5 α - β -androstane-3 α -ol-17-one glucuronides, 5 α -estrane-3 α -ol-17-one glucuronide and deuterium-labelled analogues. *J. Mass Spectrom.* 2001; 36: 159.
5. Thevis M, Opfermann G, Schmickler H, Schänzer W. Mass spectrometry of steroid glucuronide conjugates. II. Electron impact fragmentation of 3-keto-4-en- and 3-keto-5 α -steroid-17-O- β glucuronides and 5 α -steroid-3 α -17 β -diol 3- and 17-glucuronides. *J. Mass Spectrom.* 2001; 36: 998.
6. Kuuranne T, Aho O, Vehermo M, Elovaara E, Kostainen R. Enzyme-assisted synthesis and structure characterization of glucuronide conjugates of methyltestosterone (17 α -methylandroster-4-en-17 β -ol-3-one) and nandrolone (estr-4-en-17 β -ol-3-one) metabolites. *Bioconjugate Chem.* 2002; 13: 194.
7. Kuuranne T, Kurkela M, Thevis M, Schänzer W, Finel M, Kostainen R. Glucuronidation of anabolic androgenic steroids by recombinant human UDP-glucuronosyltransferases. *Drug Metab. Dispos.* 2003; 31: 1117.

Francesco Molinari, M. Gabriella Abate, Roberto Alconi, Monica Mazzanti, Francesco Rossi, Francesco Boini

Urine stability, steroid profile and T/E ratio: towards an index of sample degradation

Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Roma, Italy

Introduction

The last revision of the WADA list reduced the threshold value for an elevated testosterone/epitestosterone (T/E) concentration ratio from 6 to 4. The WADA also released a technical document [1], which calls to assess sample degradation. A preliminary retrospective evaluation of the samples received in the summer months of the last three years confirmed the empirical evidence that the degradation of the sample is often associated to an increase of T/E value, and that this pattern, especially now that the threshold of the T/E ratio has been reduced, can generate an additional workload for the laboratories.

We have tried to identify one or more suitable markers of sample degradation that, based on the data obtained by other antidoping laboratories [2-6], could be evaluated directly, possibly at the screening stage, reducing the need for additional confirmation and/or quantification procedures of endogenous steroid hormones. Different potential markers of urine degradation (pH, metabolic by-products, deconjugated steroids and the variation of the concentration of testosterone, epitestosterone, DHT and DHEA in both total and free fraction) were considered; particularly, the effect of the storage temperature and of the urinary pH on the variation of the concentration of representative endogenous steroids, in both free and conjugated fraction, was considered, with the aim of verifying whether it would be possible to understand, directly from the screening procedures for the steroid hormones in the total and in the free fraction, whether a sample is to be considered degraded, thus avoiding unnecessary, time-consuming confirmation analysis. The significance of the proposed parameters was evaluated reconsidering all the data on the steroid fraction obtained on more than 2000 samples received by our laboratory in the period May-September 2004.

EXPERIMENTAL SECTION

In all experiments the urines underwent the screening procedures for both total and free anabolic steroids and analysed by GC/MS. The relative concentrations of the following steroids (glucuronate+free fraction) were measured: testosterone (Testo), epitestosterone (epiT), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), 5 α -androstanedione and 5 β -androstanedione.

Constant temperature study

Experiments have been carried out on 10 different pools of urines, collected for two days. One aliquot of 3 mL of each pool was immediately taken for endogenous steroids analysis (free and conjugated fraction). Each pool was divided in four groups, stored at different temperatures (-20 °C, 4 °C, 25 °C and 37 °C) for 20 days.

Fixed pH study

Experiments have been carried out on 6 different pools of urines, collected for two days. One aliquot of 3 mL of each pool was immediately taken for steroids analysis (free and conjugated fraction), then each pool of urine was divided in three groups (two pool each groups) and stored at 25 °C and 37 °C and at pH 5, 7 and 9. The pH values were checked daily, and, if necessary, adjusted. The characterization of the samples was carried out following the degradation of a non-buffered group of samples, analyzed concurrently.

Reference standards

The standards were obtained by NARL-Australia (testosterone, epitestosterone), and by Sigma Aldrich (dehydroepiandrosterone, dihydrotestosterone, 5 α -androstanedione and 5 β -androstanedione).

Analytical procedure

To 3 mL of urine, 50 μ L of internal standard (17 α -methyltestosterone), 1 mL of 0.2M phosphate buffer pH=7.4, and 30 μ L of beta-glucuronidase from *E. coli* were added and hydrolysis was performed for 1 h at 50 °C. The buffered solution was then alkalized with 1 mL of carbonate buffer and the steroids were extracted with 10 mL of tert-butylmethyl ether on a mechanical shaker for 5 minutes. After centrifugation, the ethereal layer was transferred and evaporated to dryness under vacuum; the residue was derivatized by 50 μ L of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA):NH₄LiDiethoxyethylol (1000:2:4 v/w/w) and 1 μ L of the derivatized extract was injected directly into the injection port. The sample preparation for the screening analysis of the free fraction consisted only in a liquid-liquid extraction at pH 9 and derivatization with the same reagent used for the total fraction.

Quantitation of excreted steroids (free and conjugated fraction) was performed by GC/MS on an Agilent 5890V 5973A, in electron impact (70 eV), using a 17 m fused silica capillary column cross-linked methyl silicone (HP1-1D 0.20 mm, film thickness 0.11 μ m). The carrier gas was helium (flow rate: 1 mL/min, split ratio 1:10), and the temperature program was as follows: 180 °C (hold 4.5 min), 3 °C/min to 230 °C, 20 °C/min to 290 °C, 30 °C/min to 320 °C; transfer line temperature: 280 °C. Acquisition was carried out in selected ion monitoring (SIM) of the following fragments: m/z 432 for testosterone, epitestosterone and DHEA; m/z 275 for 5 α -androstanedione and m/z 417 for 5 β -androstanedione. All values of urine concentration were calculated by the peak areas of the detected signals relative to the internal standard methyltestosterone (m/z 301). For calibration of the GC/MS instrument, the following reference mixtures were used (table 1 and table 2). All experimental data are reported in the figures 1-11.

Table1: Methanolic solutions

| Compound | Internal code | CalG1 50uL Conc. ng/mL (3 mL urine) | CalG2 50uL Conc. ng/mL (3 mL urine) |
|----------------------------|---------------|---|---|
| Testosterone | Test2-001 | 10 | 40 |
| Epitestosterone | Epi-002 | 10 | 40 |
| Androsterone | H047-002 | 500 | 1000 |
| Epiandrosterone | H080-001 | 500 | 1000 |
| DHEA | DHEA-001 | 10 | 40 |
| DHT | DHT-002 | 10 | 20 |
| 5 α Androstanedione | 5ADIONE-002 | 10 | 40 |
| 5 β Androstanedione | 5BDIONE-001 | 10 | 40 |
| Androstenedione | ASTB-001 | 10 | 20 |

Table2: Blank urine spiked with the target compounds

| Compound | Internal code | USP 1 Conc. ng/mL (3 mL urine) | USP 2 Conc. ng/mL (3 mL urine) |
|----------------------------|---------------|--------------------------------------|--------------------------------------|
| Testosterone | Test2-001 | 10 | 40 |
| Epitestosterone | Epi-002 | 10 | 40 |
| Androsterone | H047-002 | 500 | 1000 |
| Epiandrosterone | H080-001 | 500 | 1000 |
| DHEA | DHEA-001 | 10 | 40 |
| DHT | DHT-002 | 10 | 20 |
| 5 α Androstanedione | 5ADIONE-002 | 10 | 40 |
| 5 β Androstanedione | 5BDIONE-001 | 10 | 40 |
| Androstenedione | ASTB-001 | 10 | 20 |

RESULTS AND DISCUSSION

Influence of the temperature

From the data reported in Figures 1-3 it can be concluded that the most relevant parameters involved - and detectable - in the urine degradation process are the followings:

- formation (first in the conjugated fraction and then in the free fraction) of 5 α -androstanedione and 5 β -androstanedione: the urinary concentrations of these substances increase quickly during storage at 37 °C, more slowly at 25 °C. No difference (as far as the steroid profile is concerned) was detected, over a 20-day period, between 4 °C and - 20 °C;
- increased concentration of testosterone, epitestosterone, DHEA and DHT in the free fraction, recorded when the urine was stored at 37 °C;
- rapid increase and then decrease of DHT and DHEA in the conjugated fraction;
- increase of the pH value, recorded when the urine was stored either at 37 °C or at 25 °C.

Influence of pH

Figures 4-7 show that the effect of pH can be summarized as follows:

- the degradation is very rapid at 37 °C, slower but still pronounced at room temperature, while no difference (as far as the steroid profile is concerned) was detected between 4 °C and - 20 °C on a 30-day period;
- the pH value appears to be critical for the degradation process, i.e. at 37 °C: the process is very slow at pH 5.0. At pH 7.0 the process is very fast;
- the degradation is still quick at pH 9.0, but it goes on for a short time.

Parameters validation

In this phase we have studied ten pools of urine, with the aim of fixing a tentative cut-off value for the two most reliable markers of urine degradation, i.e. 5 α -androstanedione and 5 β -androstanedione. Data from Figures 8-11 show that when the percentage of epitestosterone and testosterone in the free fraction is higher than the 5% of the conjugated fraction, the value of 5 α -androstanedione and 5 β -androstanedione in the total fraction is around 7 and 20 ng/mL respectively. To validate this hypothesis we have reconsidered all the "A" samples (total: 2965) analyzed in our laboratory during the summer period (from May to September 2004).

The results of this retrospective study were the followings:

- 247 of these samples were found to match our degradation criteria;
- all those samples (27) with a T/E > 6, and subsequently found to be degraded, matched our degradation criteria;

reconsidering the new limit (T/E > 4), 138 had a T/E ratio (from the screening) imposing a confirmation analysis, and 43 (31%) could be considered degraded on the basis of the limits proposed for the screening analysis (TMS-derivatives, total fraction), without running any confirmation.

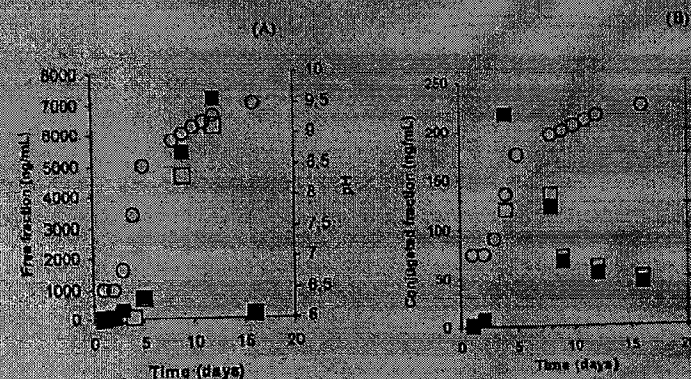


Figure 1. Variation as a function of time of the urinary concentration of 5 α -androstanedione (□), 5 β -androstanedione (■) and of the urinary pH (○), at 37 °C, both in the free (A) and in the conjugated (B) fraction.

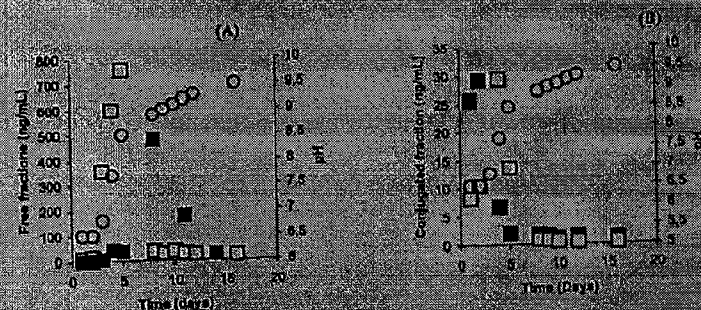


Figure 2. Variation as a function of time of the urinary concentration of DHT (□), DHEA (■) and of the urinary pH (○), at 37 °C, both in the free (A) and in the conjugated (B) fraction.

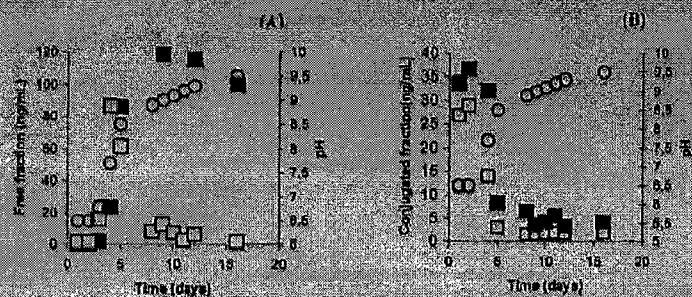


Figure 3. Variation as a function of time of the urinary concentration of: Testosterone (□), Epitestosterone (■) and of the urinary pH (○), at 37 °C, both in the free (A) and in the conjugated (B) fraction.

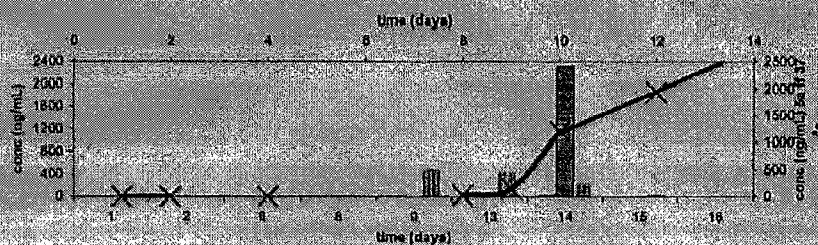


Figure 4. Variation as a function of time of the urinary concentration of 5α-androstenedione (free fraction) at 37 °C, at pH 5 (black), 7 (dashed), 9 (grey) and in non-buffered pool (x).

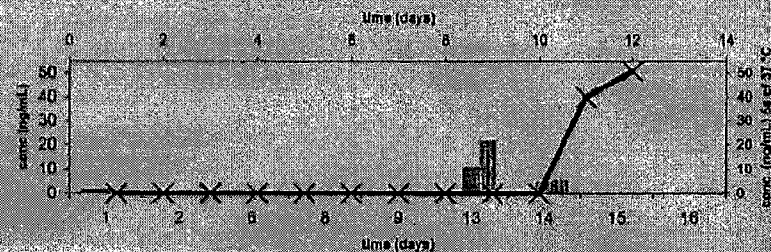


Figure 5. Variation as a function of time of the urinary concentration of 5α-androstenedione (conjugated fraction) at 37 °C, at pH 5 (black), 7 (dashed), 9 (grey) and in non-buffered pool (x).

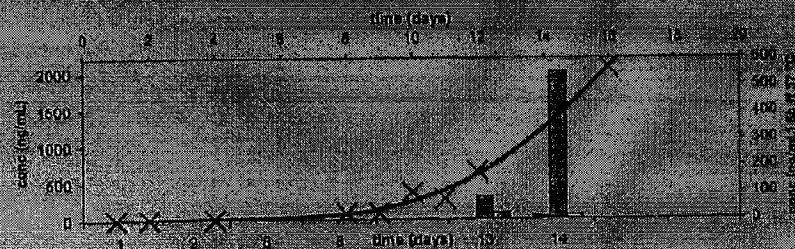


Figure 6. Variation as a function of time of the urinary concentration of 5β-androstenedione (free fraction) at 37 °C, at pH 5 (black), 7 (dashed), 9 (grey) and in non-buffered pool (x).

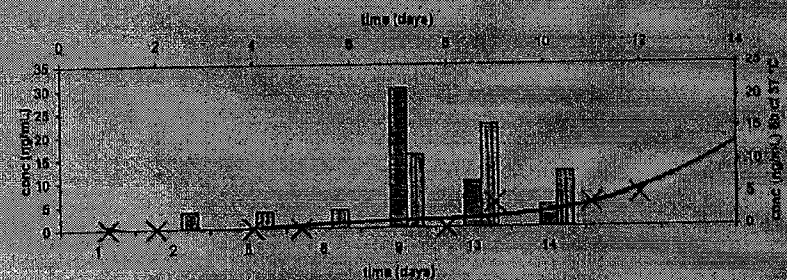


Figure 7. Variation as a function of time of the urinary concentration of 5β-androstenedione (conjugated fraction) at 37 °C, at pH 5 (black), 7 (dashed), 9 (grey) and in non-buffered pool (x).

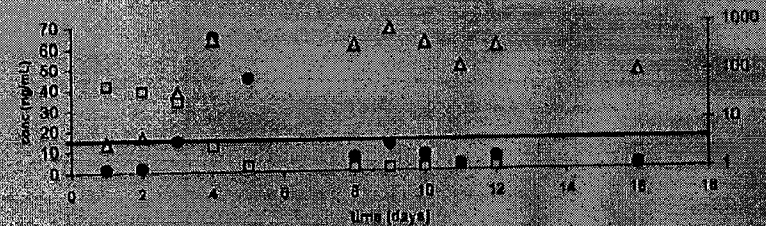


Figure 8. Variation as a function of time of the urinary concentration of testosterone in the free fraction, conjugated fraction and % free fraction/conjugated fraction.

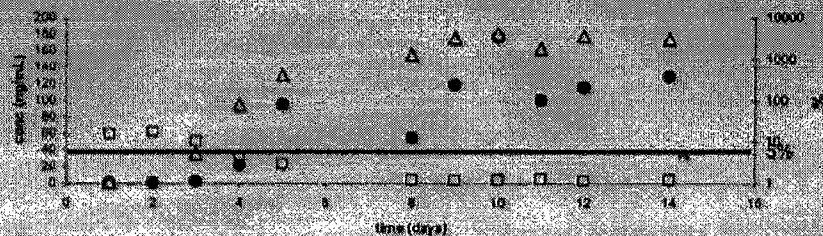


Figure 9. Variation as a function of time of the urinary concentration of epitestosterone in the
 • free fraction, □ conjugated fraction and Δ % free fraction/conjugated fraction

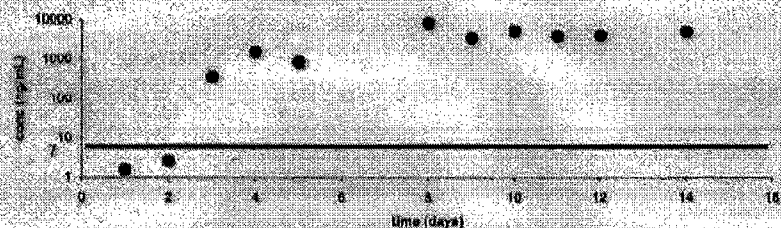


Figure 10. Variation as a function of time of the urinary concentration of 5α-androstenedione in the total fraction.

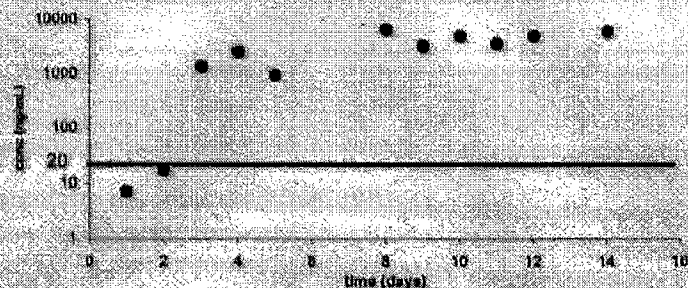


Figure 11. Variation as a function of time of the urinary concentration of 5β-androstenedione in the total fraction.

CONCLUSIONS

- The lowering of the T/E ratio value from 5 to 4 increases a useful evaluation of biological markers of doping degradation (corresponding, according to ref. [1] to a testosterone and epitestosterone concentrations ratio free/conjugated = 5%), the full quantitative confirmation in the free and total fraction of the testosterone and epitestosterone concentration may exceed the overall capacity of the laboratory (especially in the summer months).
- If the screening "IVA" (TMS-derivatives, free fraction) is performed, the simple presence of 5α-androstenedione and 5β-androstenedione is a reliable index of sample degradation: a window in the screening macro is easily added and monitored (presence/absence).
- If only the screening "IVA" (TMS-derivatives, total fraction) is performed, the concentration of 5α-androstenedione and 5β-androstenedione in the total fraction above a confidence threshold value (respectively 7 ng/mL and 40 ng/mL, respectively) is a reliable index of sample degradation.
- Alternatively, the concentration of 5α-androstenedione and 5β-androstenedione can be estimated by the height ratio 5α/ISTD and/or 5β/ISTD (in our case deuterated epitestosterone). The instrumental stability and repeatability of these parameters suggest that the laboratory internal threshold can represent a valid index for the preliminary assessment of sample degradation.

ACKNOWLEDGEMENTS

This work has been supported in part by a Research Grant of the Italian Department of Health ("Ministero della Salute, Commissione per la vigilanza sul doping e sulla tutela sanitaria delle attività sportive"). The authors also wish to thank Raffaella Stinchelli for her technical support.

REFERENCES

- [1] The World Antidoping Agency, "Reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids", Technical Document TD2004EAAS, May 30th 2004 (available on the website at <http://www.wada-ama.org/en/dynamic/ch27/pageCategoryId=372>).

- [2] R. De La Torre, X. De La Torre, J. Segura, M.T. Samoyers, R. Ventura, J.M. Torres, C. Alla, T. Baró, U. Mareck-Engelke (Eds), Recent advances in doping analysis, Sport und Buch Strauß, Köln (1998) pp. 223-236
- [3] U. Mareck-Engelke, H. Geyer and W. Schänzer, U. Mareck-Engelke (Eds), Recent advances in doping analysis, Sport und Buch Strauß, Köln (1997) pp. 51-59
- [4] H. Geyer and W. Schänzer, U. Mareck-Engelke and M. Donike in M. Donike, H. Geyer, A. Goltzmann and U. Mareck-Engelke (Eds), Recent advances in doping analysis, Sport und Buch Strauß, Köln (1996) pp. 95-113
- [5] C. Ayoite, D. Goudreau, A. Charlebois, (1996), *J. Chromatogr. B*, 687, 3-25
- [6] DH. Catlin, D. Cowan, R. De La Torre, M. Donike, D. Fraise, H. Ofiebro, CK. Halton, B. Starcevic, M. Becchi, X. De La Torre, H. Norli, H. Geyer, CJ Walzer (1996), *J. Mass Spectrom.*, 31, 397-402.

Monica Mazzarino, Maria Cristina Braganò, Francesco Donati, Francesco Botta

The effect of the oral administration of propyphenazone on the urinary concentration of testosterone and 19-nortestosterone metabolites

Laboratorio Antidoping, Federazione Medico-Sportiva Italiana, Roma, Italy

INTRODUCTION

Propyphenazone belongs structurally to the group of pyrazolone derivatives and therapeutically to non-steroidal anti-inflammatory drugs (NSAID). It has also analgesic effects. It is generically used for the treatment of fever, pain during flu or cold and after vaccination [1-2]. The drug is not prescribed often nowadays because of the many significant side effects; the normal dose is 150-300 mg 1 to 3 times per day. Some athletes use pharmaceutical formulations containing propyphenazone during multi-days events to suppress pain.

This study originates from the observation of some unknown peaks detected in a considerable number of samples by the screening procedure for the anabolic steroids. The spectrum of these peaks corresponded to the propyphenazone metabolites (Figure 1). The same samples showed an unusually altered steroid profile (Table 1).

Table 1 Steroid profile of some representative real samples, found to contain propyphenazone metabolites

| Substance | Sample 1 (ng/ml) | Sample 2 (ng/ml) | Sample 3 (ng/ml) | Normal range (ng/ml) |
|--|------------------|------------------|------------------|----------------------|
| Testosterone | 168.3 | 21.8 | 55.3 | 26-150 |
| Epiandrosterone | 111.6 | 17.5 | 24.1 | 30-150 |
| Androsterone | 152.7 | 182.3 | 18.8 | 900-2000 |
| Etiocholanolone | 317.2 | 124.7 | 4.1 | 900-2000 |
| 11 β -OH-androsterone | 2.3 | 12.3 | 2.3 | 15-50 |
| 11 β -OH-etiocholanolone | 6.3 | 7.3 | 1.4 | 10-100 |
| 3 α -androstane-5 α 17 β -diol | 47.4 | 18.3 | 4.3 | 10-200 |
| 3 β -androstane-5 α 17 β -diol | 212.4 | 73.7 | 109.6 | 10-500 |

Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (4)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1997

H. GEYER, U. MARECK-ENGELKE, W. SCHÄNZER, M. DONIKE:
The Cologne Protocol to Follow up High Testosterone / Epitestosterone Ratios
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in
doping analysis (4). Sport und Buch Strauß, Köln, (1997) 107-125

The Cologne protocol to follow up high testosterone/epitestosterone ratios

Institut für Biochemie, Deutsche Sporthochschule Köln, Germany

This lecture was held at the 11th and 12th Cologne Workshop on Dope Analysis 1993 and 1994.

Introduction

The most important parameter for the detection of doping with exogenous testosterone is an elevated ratio testosterone/epitestosterone (1, 2). Before a dope control sample is declared positive for testosterone, it has to be proven, according to the rules of the IOC (3), that the elevated ratio testosterone/epitestosterone is not natural. Therefore we have developed in cooperation with the Antidoping Commission of the International Cycling Federation (UCI) a complex protocol to follow up high testosterone/epitestosterone values. The main intention of this protocol is the prevention of false positive results.

The knowledge of the „normal“ steroidprofile as basis for the detection of doping with testosterone

The basis for the detection of doping with testosterone and other endogenous steroids is the knowledge of the „normal“ steroid profile, i.e. population based reference ranges of endogenous steroids. Therefore we have analysed within our screening procedure for anabolic steroids steroidprofiles of dope control urines for several years. All samples were analysed and quantified with the same method, which was described in detail several times (4, 5). In table 1 are presented the endogenous steroids, which are monitored for steroidprofiling in the Cologne laboratory.

Tab. 1: Retention times, retention indices and ion traces of the endogenous steroids which are monitored for steroidprofiling in the Cologne laboratory. Also added are the internal standards (all steroids as per-trimethylsilyl derivatives).

| Substanz | Abbreviations | RT | Index | m/e |
|--|--------------------------|-------|-------|-----|
| 5 β -androstane-3 α ,17 α -diol | | 8.64 | 2419 | 241 |
| 5 β -androstane-3 β ,17 α -diol | | 8.99 | 2437 | 241 |
| 5 α -androstane-3 α ,17 α -diol | | 9.12 | 2433 | 241 |
| 5 β -androstandion | | 9.19 | 2437 | 432 |
| d ₅ -androsterone | d5-AND | 10.28 | 2501 | 439 |
| androsterone | AND | 10.36 | 2506 | 434 |
| d ₄ -etiocholanolone | d4-ETIO | 10.46 | 2511 | 438 |
| etiocholanolone | ETIO | 10.54 | 2515 | 434 |
| 5 α -androstane-3 α ,17 β -diol | 5 α A3 α D | 10.68 | 2522 | 241 |
| 5 β -androstane-3 α ,17 β -diol | 5 β A3 α D | 10.82 | 2529 | 241 |
| dehydroepiandrosterone | DHEA | 11.66 | 2572 | 432 |
| epiandrosterone | EPIAND | 11.78 | 2578 | 434 |
| 5 α -androstandion | | 12.11 | 2595 | 432 |
| 5-androsten-3 β ,17 β -diol | | 12.13 | 2596 | 434 |
| 5 α -androstane-3 β ,17 β -diol | 5 α A3 β D | 12.15 | 2597 | 421 |
| d ₃ -epitestosterone | d3-EPIT | 12.15 | 2597 | 435 |
| epitestosterone | EPIT | 12.19 | 2599 | 432 |
| dihydrotestosterone | DHT | 12.41 | 2610 | 434 |
| 4-androstendion | | 12.85 | 2633 | 430 |
| d ₃ -testosterone | d3-TEST | 12.99 | 2640 | 435 |
| testosterone | TEST | 13.03 | 2642 | 432 |
| 11 β -hydroxy-androsterone | 11OHAN | 13.39 | 2660 | 522 |
| 11 β -hydroxy-etiocholanolone | 11OHET | 13.65 | 2673 | 522 |
| methyltestosterone | MTEST | 14.82 | 2733 | 446 |
| pregnanediol* | | 15.37 | 2761 | 117 |
| pregnanetriol** | | 15.98 | 2792 | 255 |

* 5 β -pregnane-3 α ,20 α -diol

** 5 β -pregnane-3 α ,17 α ,20 α -triol

The descriptive statistics and the calculated population based reference ranges of the steroidprofile parameters are presented in table 2-5. All values and details of the statistical calculation (reference population, reference sample group etc.) are published in the thesis of S. RAUTH (6). The calculation of the population based reference ranges were performed according to a concept and to recommendations of the International Federation of Clinical Chemistry (IFCC). All concentrations are corrected to a urine density of 1.020 g/cm³.

Tab. 2: Descriptive statistics of the concentration of endogenous steroids and the concentration of the reference sample group men. (6)

| concentrations | N | \bar{x} [ng/ml] | 10.Perc. [ng/ml] | 90.Perc. [ng/ml] | \bar{x} [ng/ml] | s [ng/ml] | skewness | excess |
|----------------|------|----------------------|---------------------|---------------------|----------------------|--------------|----------|---------|
| AND | 5101 | 2671 | 1322 | 4986 | 2975 | 1545.23 | 1.31 | 2.94 |
| ETIO | 5101 | 2032 | 1020 | 3797 | 2279 | 1209.92 | 1.58 | 4.88 |
| EPIT | 5095 | 27.7 | 9.2 | 70.6 | 35.7 | 30.03 | 2.77 | 15.49 |
| TEST | 5100 | 36.8 | 6.3 | 88.3 | 44.6 | 37.94 | 2.78 | 20.06 |
| Adiol | 5088 | 49.6 | 22.1 | 108.3 | 60.4 | 44.87 | 3.84 | 33.30 |
| Bdiol | 5088 | 128.1 | 38.0 | 342.8 | 166.5 | 141.79 | 2.28 | 9.26 |
| Pregnd | 5088 | 279.9 | 118.0 | 616.7 | 335.0 | 231.51 | 2.39 | 11.82 |
| ratios | N | \bar{x} | 10.Perc. | 90.Perc. | \bar{x} | s | skewness | excess |
| AND/ETIO | 5283 | 1.32 | 0.77 | 2.22 | 1.43 | 0.60 | 1.21 | 2.78 |
| TEST/EPIT | 5277 | 1.35 | 0.25 | 3.29 | 1.65 | 1.41 | 3.54 | 41.49 |
| AND/TEST | 5282 | 72.5 | 32.7 | 400.5 | 191.0 | 620.82 | 23.46 | 904.87 |
| AND/EPIT | 5283 | 94.0 | 43.0 | 244.6 | 126.7 | 110.18 | 4.03 | 34.93 |
| ETIO/TEST | 5283 | 54.9 | 24.8 | 294.8 | 143.2 | 480.82 | 26.94 | 1120.79 |
| ETIO/EPIT | 5277 | 72.9 | 30.1 | 195.3 | 99.9 | 99.06 | 5.54 | 67.61 |
| Adiol/Bdiol | 5283 | 0.41 | 0.17 | 1.03 | 0.52 | 0.41 | 3.60 | 41.22 |

Tab. 3: Descriptive statistics of the concentrations of the endogenous steroids and the concentration ratios of the reference sample group women. (6)

| concentrations | N | \bar{x} [ng/ml] | 10.Perc. [ng/ml] | 90.Perc. [ng/ml] | \bar{x} [ng/ml] | s [ng/ml] | skewness | excess |
|----------------|------|----------------------|---------------------|---------------------|----------------------|--------------|----------|--------|
| AND | 1696 | 1859 | 802 | 4199 | 2286 | 1629.40 | 2.22 | 7.99 |
| ETIO | 1696 | 1990 | 891 | 4050 | 2326 | 1548.73 | 3.15 | 27.53 |
| EPIT | 1693 | 6.0 | 2.1 | 22.1 | 10.0 | 12.72 | 4.97 | 41.95 |
| TEST | 1693 | 8.2 | 2.1 | 25.5 | 12.6 | 15.21 | 4.09 | 24.86 |
| Adiol | 1694 | 18.5 | 8.2 | 48.1 | 25.1 | 24.30 | 3.76 | 20.77 |
| Bdiol | 1694 | 64.1 | 18.1 | 190.3 | 92.5 | 92.88 | 2.70 | 10.90 |
| Pregnd | 1695 | 390.5 | 146.2 | 1320.6 | 642.5 | 884.49 | 4.98 | 36.29 |
| ratios | N | \bar{x} | 10.Perc. | 90.Perc. | \bar{x} | s | skewness | excess |
| AND/ETIO | 1742 | 0.96 | 0.58 | 1.59 | 1.04 | 0.44 | 1.47 | 4.53 |
| TEST/EPIT | 1736 | 1.34 | 0.34 | 3.87 | 1.83 | 1.82 | 3.90 | 31.34 |
| AND/TEST | 1739 | 220.6 | 82.2 | 777.0 | 449.9 | 1384.28 | 21.15 | 621.99 |
| AND/EPIT | 1742 | 304.3 | 119.0 | 657.7 | 355.4 | 230.63 | 1.45 | 3.31 |
| ETIO/TEST | 1742 | 232.1 | 90.0 | 746.3 | 462.7 | 1181.14 | 12.35 | 207.04 |
| ETIO/EPIT | 1739 | 321.5 | 109.0 | 812.9 | 399.5 | 301.30 | 1.56 | 3.06 |
| Adiol/Bdiol | 1742 | 0.31 | 0.11 | 0.84 | 0.41 | 0.36 | 4.80 | 61.35 |

Tab. 4: Reference limits of the non-parametric 95% reference range and the respective 90% confidence intervals for the reference sample group of men.

(N(concentrations)=5101, N(ratios)=5283 (6))

| concentrations | Reference limits | | 90% confidence interval | | | |
|--------------------------|------------------|------------------|-------------------------|-------|------------------------|-------|
| | 2.5% [ng/ml] | 97.5% [ng/ml] | lower limit [ng/ml] | | upper limit [ng/ml] | |
| AND | 867.2 | 6703 | 834.5 | 894.3 | 6533 | 6881 |
| ETIO | 673.7 | 5294 | 654.3 | 697.1 | 5107 | 5497 |
| EPIT | 4.9 | 112 | 4.56 | 5.39 | 106.7 | 118.4 |
| TEST | 2.06 | 137.4 | 1.87 | 2.18 | 131.4 | 143.8 |
| 5 α A3 α D | 13.85 | 166.5 | 13.15 | 14.72 | 161.4 | 173.3 |
| 5 β A3 α D | 19.63 | 550.1 | 18.44 | 21.29 | 531.3 | 572.8 |
| Pregnd | 73.2 | 951.2 | 70.6 | 77.2 | 910.7 | 984.1 |
| ratios | Reference limits | | 90% confidence interval | | | |
| | 2.5% | 97.5% | lower limit | | upper limit | |
| AND/ETIO | 0.55 | 2.869 | 0.53 | 0.57 | 2.81 | 2.93 |
| TEST/EPIT | 0.08 | 5.19 | 0.07 | 0.08 | 5.01 | 5.4 |
| AND/TEST | 22.5 | 1164 | 21.7 | 23.2 | 1052 | 1269 |
| AND/EPIT | 27.7 | 406.3 | 26.6 | 28.7 | 386.8 | 426.7 |
| ETIO/TEST | 16.7 | 819.2 | 16.3 | 17.2 | 768.6 | 905.9 |
| ETIO/EPIT | 20.5 | 345.8 | 19.92 | 21.42 | 326.8 | 362.3 |
| Adiol/Bdiol | 0.11 | 1.56 | 0.11 | 0.12 | 1.51 | 1.61 |

Tab. 5: Reference limits of the non-parametric 95% reference range and the respective 90% confidence intervals for the reference sample group of women.

(N_(concentrations)=1694, N_(ratios)=1742) (6)

| concentrations | Reference limits | | 90% confidence interval | | | |
|--------------------------|------------------|------------------|-------------------------|-------|------------------------|-------|
| | 2.5% [ng/ml] | 97.5% [ng/ml] | lower limit [ng/ml] | | upper limit [ng/ml] | |
| AND | 404.1 | 6439 | 368.8 | 458.4 | 6135 | 6964 |
| ETIO | 473.2 | 6107 | 395.2 | 503.1 | 5698 | 6564 |
| EPIT | 1.11 | 42.2 | 1.04 | 1.21 | 37.2 | 47.9 |
| TEST | 0.65 | 57.3 | 0.55 | 0.83 | 48.1 | 62.1 |
| 5 α A3 α D | 4.74 | 91 | 4.12 | 5.14 | 83.1 | 98.3 |
| 5 β A3 α D | 9.14 | 366.4 | 7.97 | 10.1 | 312.4 | 387.8 |
| Pregnd | 83.1 | 3089 | 76.9 | 89.3 | 2659 | 3382 |
| ratios | Reference limits | | 90% confidence interval | | | |
| | 2.5% | 97.5% | lower limit | | upper limit | |
| AND/ETIO | 0.42 | 2.15 | 0.39 | 0.44 | 2.03 | 2.22 |
| TEST/EPIT | 0.11 | 6.31 | 0.1 | 0.13 | 5.91 | 6.7 |
| AND/TEST | 47.9 | 2184 | 40.0 | 52.1 | 1704 | 2591 |
| AND/EPIT | 65.3 | 939.4 | 60 | 74.3 | 893.9 | 986.8 |
| ETIO/TEST | 37.0 | 2397 | 31.8 | 42.0 | 1998 | 2905 |
| ETIO/EPIT | 60.5 | 1205 | 53.1 | 65.0 | 1152 | 1289 |
| Adiol/Bdiol | 0.07 | 1.24 | 0.07 | 0.08 | 1.16 | 1.28 |

Parameters for the detection of doping with testosterone

The application of exogenous testosterone leads to characteristic changes of the normal steroidprofile (1). The most obvious change is the **increase of the ratio TEST/EPIT**. A value of this parameter above the upper limit of the reference range, i.e. higher than 6, indicates a suspicion for testosterone doping. Beside the TEST/EPIT ratio, two other parameters are important for the detection of testosterone doping. These parameters are a **decreased ratio of AND/TEST** and an **increased concentration of TEST (cTEST)**.

If all three parameters are outside of the limits of the reference values (see table 6) and other influences, e.g. bacterial activities, can be excluded, there is a high probability, that the unnormal steroidprofile is caused by a testosterone application. But because the T/E ratio is the most sensitive parameter for the detection of doping with testosterone, a suspicion is already given, if the T/E ratio is higher than the upper reference limit.

Tab. 6: Parameters of the steroidprofile, which make a urine suspicious for an application of exogenous TEST.

| Parameter | Men | Women |
|------------------|-------|-------|
| TEST/EPIT | > 6 | > 6 |
| AND/TEST | < 20 | < 40 |
| c TEST [ng/ml] * | > 130 | > 60 |

* concentration corrected to a urine density of 1.020

To study, if the high T/E ratio is natural, caused by a testosterone application or caused by other influences, e.g bacterial, pathological or analytical influences, we start with the following protocol.

Screening results -Search for other factors which may increase the ratio TEST/EPIT

If there was found a ratio TEST/EPIT > 6 we look first in our screening results for other factors, which may lead, beside the TEST application, to increased TEST/EPIT ratios or change the other testosterone related parameters AND/TEST and cTEST. In our screening method we have included some controls to recognize these factors:

Mistakes in the sample preparation and changes of the GC/MS conditions may change the TEST/EPIT ratio and the other testosterone related parameters. This is controled by the use of the internal standards d3-TEST, d3-EPIT and d4-ETIO. The d3-TEST and d3-EPIT are

added to each sample in a ratio of 6:1 (90 ng/ml / 15 ng/ml) so that the ratios TEST/EPIT can be corrected (7).

Bacterial activities in the urine can be recognized by the presence of 5 α - and 5 β -androstandion (4), which are monitored in our screening procedure. Also high amounts of DHT are found. The AND/TEST ratios are often low. These changes of the steroidprofile are mostly connected with high pH values. Further studies of such samples have shown, that in all of these samples high amounts of the steroid glucuronides are hydrolysed, and that the urine contains different enzyme activities as 3-hydroxy-steroid-dehydrogenase activity or steroid- Δ -isomerase etc. which can transform unconjugated androsterone to 5 α -androstandion or unconjugated 5-androstendiol to testosterone (5). In these cases, the steroidprofile parameters are worthless and we stop the follow up protocol. We inform the federation about the presence of bacterias, which are mostly caused by insuitable transport or storage conditions and recommend to repeat the dope controls and to improve the transport and storage conditions.

Bacterial activities in the phosphate buffer used for the hydrolysis can not be recognized from our screening chromatograms. We control such side activities in the hydrolysis step by spiking a buffer solution with 5 μ g of 5-androstene-3 β ,17 β -diol and DHEA (8). If TEST or 5-androstendion is found after incubation of 1 hour at 50° C, the sample preparation has to be repeated.

We prevent such side activities by heating the buffer for several minutes before use (8).

Incomplete hydrolysis leads to a low and therefore suspicious ratio AND/TEST because AND-glucuronide is hydrolysed slower than TEST-glucuronide (5). We control the hydrolysis with the internal standard [2,2,3,4,4-²H₅]-androsterone-D-17-glucuronide, which is added in an amount of 500 ng/ml to each sample. In case of an incomplete hydrolysis, we repeat the sample preparation.

Incomplete derivatisation may lead to wrong steroidprofile values, e.g. to low AND/TEST values. The derivatisation is controlled by the monitoring of the ion 272 (molecular ion -90) for AND and ETIO, mono-TMS. If these two substances are detected, the derivatisation is

incomplete and we add to the derivatised sample (ca. 80 µl) once more 20 µl of derivatisation reagent with a higher concentration of the catalyst NH_4I (2%).

If we have excluded all above mentioned factors for the high TEST/EPIT ratio we start with the confirmation of the sample.

Confirmation of suspicious TEST/EPIT samples

Our procedure for the confirmation of samples with high TEST/EPIT ratios was exactly described by NOLTEERNSTING et al. (7).

The most important points are:

Reproducibility

To examine the reproducibility of the method, we prepare three aliquots of the sample which are injected twice. The variation coefficient of the TEST/EPIT ratios should be better than 5%. The mean of the TEST/EPIT values of the replicates minus three times the standard deviation should result in a value higher than 6.

Correction with deuterated standards

For the correction of the ratios TEST/EPIT the internal standards d3-TEST/d3-EPIT 6:1 are added to each sample.

Full scan spectrum of TEST and EPIT

Additional to the 3 aliquots we prepare one aliquot without internal standard to achieve full scan spectra of TEST and EPIT. This is done to show, that no other substance is coeluted with TEST or EPIT.

Increased urine volume

The used urine volume is increased to achieve a good GC signal especially for EPIT.

Separation of the free fraction

Before the hydrolysis, the free steroids are separated by extraction with ether. By this step, we prevent false positive results by unconjugated, bacterial produced, TEST (5).

Heating of the buffer

To avoid testosterone producing side activities from the phosphate buffer (8) during the hydrolysis, we boil the phosphate buffer for several minutes before the use.

*Hydrolysis with β -glucuronidase from *E.coli**

For the hydrolysis of the glucuronides we only use β -glucuronidase from *E. coli*, because we have never observed testosterone producing side activities in this enzyme preparation. Such activities can be found in the β -glucuronidase/ aylsulfatase of *Helix Pomatia* preparations (9).

n-pentane extraction

After hydrolysis the extraction of the steroids is performed with n-pentane instead of tert. butylmethyl ether. This is an additional clean up step to reduce the biological background for the detection of TEST and EPIT (10).

Changed MS parameters

For the MS detection is used a SIM method with only 5 ions. The molecular ions of the bis-TMS derivatives of TEST, EPIT, d3TEST and d3EPIT are registered with dwell times of 200 msec (7).

Measurement of LH, FSH and HCG

As proposed by KICMAN et al.(11) and COWAN et al.(12) we analyse also the gonadotropines LH, FSH and HCG. Of most importance is the ratio LH/TEST.

Measurement of ethanol

Several studies have shown that the application of ethanol may increase the TEST/EPIT ratio and decrease the AND/TEST ratio (13). Additionally we have found, that these changes of the steroid profile are always connected with the presence of ethanol in urine (13). Therefore we analyse samples with elevated TEST/EPIT ratios also for ethanol by Headspace/GC.

If we find ethanol, we stop the follow up protocol and don't give the sample positive. Until now we observed such cases only in Out of Competition Controls.

If the TEST/EPIT ratio > 6 is confirmed and the additional analyses are performed, we continue with the following steps.

Information of the Federation and request for further data

We inform the federation about the high TEST/EPIT ratio and ask for retrospective (code numbers of former dope controls) or prospective data (further dope controls) . For retrospective data, which are archived in other laboratories, we recommend the federation to provide the information listed in the formsheet for high TEST/EPIT values (Fig. 1). For the decision making process, we need at least data from 4 samples of the same athlete. The statistical evaluation of the data is performed as presented in the following chapter

If the retrospective or prospective data are not sufficient for the decision (e.g. not enough data, strong variation of the data), an endocrinological study is performed.

Elevated TEST/EPIT value

hg/doku/s44/aaafe

| | |
|---------------------------|--|
| Laboratory | |
| Sample Code | |
| Laboratory Code | |
| Event | |
| Date of dope control | |
| Declaration of medication | |

Screening Results

| | |
|---|--|
| pH | |
| specific gravity [g/cm ³] | |
| androsterone [ng/ml] | |
| etiocholanolone [ng/ml] | |
| testosterone [ng/ml] | |
| epitestosterone [ng/ml] | |
| 5 α -androstane-3 α ,17 β -diol [ng/ml] | |
| 5 β -androstane-3 α ,17 β -diol [ng/ml] | |
| LH [mIU/ml] | |
| ratio of testosterone / epitestosterone (corrected) | |
| ratio androsterone / testosterone | |
| ratio androsterone / etiocholanolone | |
| ratio testosterone / LH [nmol/TU] | |

Screening method

| | |
|---|--|
| fraction (free/conjugated/combined) | |
| enzyme preparation for hydrolysis | |
| correction of ratio testosterone/epitestosterone (deuterated standards, calibration curve) | |

Confirmation results

| | |
|------------------------------------|--|
| ratio testosterone/epitestosterone | |
|------------------------------------|--|

Confirmation method

| | |
|---|--|
| fraction (free/conjugated/combined) | |
| enzyme preparation for hydrolysis | |
| extraction (n-pentane/ether) | |
| correction of ratio testosterone/epitestosterone (deuterated standards, calibration curve) | |
| mass spectrum of testosterone, epitestosterone | |

Remarks

Fig. 1: Formsheets for collection of data from other laboratories.

Endocrinological study (ES)

The aim of an ES is to calculate the subject based reference range for the TEST/EPIT ratio and the normal variation of the other TEST related steroid ratios. The basis for the judgement of the results is our knowledge about the stability of steroidprofile parameters in rest and exercise (14, 15, 16). The decision, positive or negative for the suspicious sample, is based upon a comparison of the steroidprofile values of the suspicious sample and the values of the ES.

The ES takes 2 days .During this time the athlete lives near by the laboratory. He can keep normal nutrition and training. The application of ethanol is not permitted.

Procedure

- Information of athlete about the aim and the procedure of the ES.
- Answering of a questionnaire (see Fig. 2)
- Collection of all urine fractions in a time period of at least 48 hours (ca. 13 - 15 samples according to a collection schedule (see Fig. 3).
At least 5 urine collections have to take place under observation of a member of the laboratory staff.
- Single blood sample collection (10 ml) performed by medical staff.

Analysis

| <i>Parameter</i> | <i>Single parameter</i> | <i>Analysentechnik</i> |
|--|--|------------------------|
| Steroidprofile parameters in urine (concentrations, excretion rates, ratios) | Glucuronides of androsterone, etiocholanolone, testosterone, epitestosterone, 5 α -androstan-3 α ,17 β -diol, 5 β -androstan-3 α ,17 β -diol, dihydrotestosterone, tetrahydrocortisol, 11 β -hydroxyandrosterone, 11 β -hydroxyetiocholanolone, pregnandiol, pregnantriol | GC/MSD |
| Peptide hormones in urine | LH, FSH, HCG | Enzymimmunoassay |
| Blood parameters | Testosterone, 17 α -hydroxyprogesterone (eventually esters of testosterone) | GC/HRMS |

Evaluation

- Statistical evaluation of the steroidprofile parameters and of the peptide hormone values (mean, standard deviation, coefficient of variation) for the determination of the individual reference ranges.
- Comparison of the values of the ES with the values of the suspicious sample.
- Comparison of the values of the ES with population based reference ranges.

Endocrinological study

Cologne,

| | |
|-------------|--|
| Last name: | Date of birth: |
| First name: | Sex: male <input type="radio"/> female <input type="radio"/> |
| Street: | Size [cm]: |
| City: | Weight: [kg]: |
| Country: | |

| | |
|--------------------|--|
| Federation: | Professional: <input type="radio"/> Amateur: <input type="radio"/> |
| Major Discipline: | Years of training: |
| Coach: | Training sessions per week: |
| Federation doctor: | Training hours per week: |

| Current training: | | | | | | | | | | | | | | | | |
|--|------------|------------|------------|------------|--|--|--|--|--|--|--|--|--|--|--|--|
| Current health status: | | | | | | | | | | | | | | | | |
| Current medication: | | | | | | | | | | | | | | | | |
| Former dope controls: <table><thead><tr><th>Date</th><th>City/Event</th><th>Code-Nr.</th><th>Laboratory</th></tr></thead><tbody><tr><td colspan="4"> </td></tr><tr><td colspan="4"> </td></tr><tr><td colspan="4"> </td></tr></tbody></table> | Date | City/Event | Code-Nr. | Laboratory | | | | | | | | | | | | |
| Date | City/Event | Code-Nr. | Laboratory | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | |
| Remarks: | | | | | | | | | | | | | | | | |

Fig. 2: Questionnaire for the endocrinological study

Urine Collection Schedule

Name:

| Day/Date | Collection time | Actual collection time | Volume [ml] | Remarks |
|----------|-----------------|------------------------|-------------|---|
| 1. day | 7.00 | | | |
| | 10.00 | | | observation |
| | 13.00 | | | |
| | 16.00 | | | observation |
| | 19.00 | | | |
| | 22.00 | | | |
| 2. day | 7.00 | | | |
| | 10.00 | | | observation blood sample collectionn |
| | 13.00 | | | |
| | 16.00 | | | observation |
| | 19.00 | | | |
| | 22.00 | | | |
| 3. day | 7.00 | | | |
| | 10.00 | | | observation |
| | 13.00 | | | |

Fig. 3: Urine collection schedule for the endocrinological study. Several collections take place under observation.

Data evaluation of endocrinological studies or longitudinal studies

In table 7 and 8 are presented the results of an endocrinological study of a male athlete with 12 samples. The coefficients of variation (cv in %) for the presented steroid ratios should be

within 10 % and 25 % (14,15,16). If we have higher variations, e.g. in longitudinal studies with only 3 or 4 samples, we cannot calculate the subject based reference range for the TEST/EPIT ratio. For the ratio TEST/LH we observe always variations higher than 20%.

Tab. 7: Results of an ES. Concentrations of endogenous steroids.

| # | time | AND [ng/ml] | ETIO [ng/ml] | EPIT [ng/ml] | TEST [ng/ml] |
|----|-------|----------------|-----------------|-----------------|-----------------|
| 1 | 10:15 | 6133 | 7138 | 18,6 | 83,4 |
| 2 | 13:45 | 2334 | 2580 | 6,1 | 34,5 |
| 3 | 16:05 | 2917 | 3252 | 5,9 | 35,2 |
| 4 | 19:00 | 3691 | 3930 | 9,6 | 51,2 |
| 5 | 00:05 | 2905 | 3657 | 9,0 | 40,1 |
| 6 | 10:45 | 2286 | 3103 | 7,0 | 45,9 |
| 7 | 12:15 | 3130 | 3926 | 9,0 | 67,2 |
| 8 | 16:25 | 4304 | 4582 | 13,5 | 96,3 |
| 9 | 19:15 | 2164 | 2389 | 5,5 | 36,8 |
| 10 | 22:00 | 2412 | 3201 | 7,3 | 46,4 |
| 11 | 07:35 | 2047 | 2437 | 5,2 | 36,1 |
| 12 | 10:45 | 3400 | 4525 | 10,0 | 75,2 |

Tab. 8: Results of an ES. Ratios of endogenous steroids and LH values. The ratios TEST/EPIT are corrected with the internal standards d3-TEST/d3-EPIT 6:1 (7). Presented are also the mean, standard deviation (stdev) and coefficient of variation in percent (cv %).

| # | time | TEST/ EPIT corr. | AND/ TEST conc | AND/ EPIT conc | AND/ ETIO conc | LH [mIU/ml] | TEST/LH [nmol/IU] |
|-------|-------|------------------------|----------------------|----------------------|----------------------|----------------|----------------------|
| 1 | 10:15 | 4,6 | 73,5 | 330,1 | 0,9 | 9,4 | 30,9 |
| 2 | 13:45 | 4,7 | 67,7 | 382,6 | 0,9 | 2,1 | 57,6 |
| 3 | 16:05 | 5,6 | 82,9 | 494,4 | 0,9 | 3,1 | 39,4 |
| 4 | 19:00 | 4,5 | 72,1 | 386,5 | 0,9 | 1,9 | 91,6 |
| 5 | 00:05 | 4,7 | 72,4 | 322,7 | 0,8 | 2,7 | 51,6 |
| 6 | 10:45 | 5,9 | 49,8 | 326,1 | 0,7 | 6,9 | 23,2 |
| 7 | 12:15 | 6,9 | 46,6 | 347,7 | 0,8 | 8,0 | 29,1 |
| 8 | 16:25 | 6,8 | 44,7 | 318,8 | 0,9 | 7,8 | 42,7 |
| 9 | 19:15 | 5,8 | 58,8 | 392,1 | 0,9 | 4,6 | 27,9 |
| 10 | 22:00 | 5,7 | 52,0 | 330,4 | 0,8 | 6,7 | 24,0 |
| 11 | 07:35 | 6,1 | 56,7 | 392,8 | 0,8 | 5,8 | 21,4 |
| 12 | 10:45 | 6,9 | 45,2 | 340,7 | 0,8 | 6,4 | 40,8 |
| mean | | 5,7 | 60,2 | 363,7 | 0,8 | 5,5 | 40,0 |
| stdev | | 0,9 | 13,1 | 50,2 | 0,1 | 2,5 | 19,9 |
| cv % | | 15,9 | 21,7 | 13,8 | 8,9 | 46,2 | 49,7 |

From mean and standard deviation (stdev) of the corrected TEST/EPIT ratios we calculate the limits of the subjected based reference range according to the formula (17):

- upper limit of subject based reference range = mean + 3 x stdev
- lower limit of subject based reference range = mean - 3 x stdev

In figure 4 are presented the single TEST/EPIT values of an ES and the corresponding reference limits

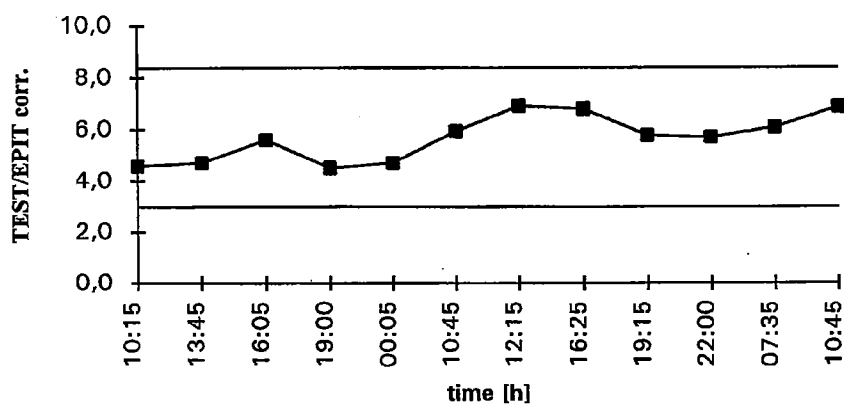


Fig. 4: TEST/EPIT values of an ES and corresponding limits of the subject based reference range (see table 8).

Blood samples in the ES

During the ES we take 10 ml of blood. We take this sample to receive additional TEST related information, e.g. about the ratio TEST/17 α - hydroxyprogesteron. The analysis is performed according to the GC/HRMS method described by HORNING et al. (18).

With this blood sample we have also the possibility, to prove an application of TEST during the ES by the detection of TEST-esters in the plasma.

Ketoconazole Test during the ES

A Ketoconazole test is an effective method for the differentiation between a natural elevated TEST/EPIT and a TEST abuse by an athlete (19). Such a test may be useful to detect a TEST application during the ES. But because of possible health risks by side effects of this drug, we hesitate to perform this test.

Interpretation of the results of an ES or a longitudinal study and recommendation to the federation

If the TEST/EPIT values and the other TEST related ratios of the suspicious samples lie within the subject based reference range and the variations of the ES values, we recommend to give the sample negative. In this case, we have a natural elevated TEST/EPIT ratio. The comparison of the ES values with the population based reference ranges allows to recognize the reason for the increased TEST/EPIT ratio. Most often a low EPIT concentration is responsible for the natural elevated TEST/EPIT ratio. Such a case is presented in tables 7 and 8. A low EPIT case is characterised by the following parameters:

- Concentration of EPIT near or outside the lower limit of the reference range
- Ratio AND/EPIT near or outside the upper limit of the reference range
- Ratio AND/TEST near the mean and median of this parameter
- Concentration of TEST near the mean and median of this parameter.

If we have a low EPIT case, we recommend to the federation to give a certification (naturally elevated T/E ratio) to the athlete. The certification should have the following consequences: In case a laboratory shows a "positive " result for TEST related parameters, the Antidoping Commission of the federation should be informed at once and before disciplinary measures be taken against the athlete. The decision positive or negative should be made only after the comparison of the suspicious values with the archived values of the athlete.

References:

1. Donike, M., Bärwald, K.-R., Klostermann, K., Schänzer, W., Zimmermann, J.: Nachweis von exogenem Testosteron. in: H.Heck, W.Hollmann, H.Liesen, R.Rost (eds.) Sport: Leistung und Gesundheit., Deutscher Ärzte Verlag Köln, (1983) 293.
2. Zimmermann, J.: Untersuchung zum Nachweis von exogenen Gaben von Testosteron. Thesis. Deutsche Sporthochschule Köln. 1986
3. IOC Medical Code, 1995.
4. Donike, M., Ueki, M., Kuroda, Y., Geyer, H., Nolteernsing, E., Rauth, S., Schänzer, W., Schindler, U., Völker, E., Fujisaki, M.: Detection of dihydro-testosterone (DHT) doping - Alterations in the steroid profile and reference ranges for DHT and its 5 α -metabolites. J. Sports.Med.Phys.Fitness, 35 (1995) 235-50.
5. Geyer, H., Schänzer, W., Mareck-Engelke, U., Donike, M.: Factors influencing the steroid profile. in: M.Donike, H.Geyer, A.Gotzmann, U.Mareck-Engelke (Eds.) Recent advances in doping analysis (3) Sport und Buch Strauß, Köln (1996) 95-114.
6. Rauth, S.: Referenzbereiche von urinären Steroidkonzentrationen und Steroidquotienten. Ein Beitrag zur Interpretation des Steroidprofils in der Routinedopinganalytik. Dissertation, Deutsche Sporthochschule Köln (1994).
7. Nolteernsing, E., Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M.: Standardisation of the T/E-determination by deuterated internal standards. In: M. Donike, H.Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995) 113-120.
8. Geyer, H.: Die gas-chromatographisch/massenspektrometrische Bestimmung von Steroidprofilen im Urin von Athleten. Dissertation, Deutsche Sporthochschule Köln (1990).
9. Kuoppasalmi, K., Leinonen, A., Kajalainen, U.: Detection of exogenous testosterone in doping analysis: methodological aspects. In: Sports medicine and exercise science. Proceedings of the Olympic scientific congress. Eugene, Oregon, 1984.
10. Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M.: Simple purification of urine samples for improved detection of anabolic and endogenous steroids. In: M. Donike, H.Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.) Recent advances in doping analysis. Sport und Buch Strauß, Köln (1994) 97-104.
11. Kicman, A.T., Brooks, R.V., Collyer, S.C. et al.: Criteria to indicate testosterone administration. Brit.J.Sports Med. 24 (1990) 253-264.
12. Cowan, D.A., Kicman, A.T., Walker, C.J., Wheeler, M.J.: Effects of administration of human chorionic gonadotrophin on criteria used to assess testosterone administration in athletes. J. of Endocrinology 131 (1991) 147-154.
13. Mareck-Engelke U., Geyer H., Schindler U., Flenker U., Iffland R. and Donike M.: Influence of Ethanol on Steroid Profile Parameters in: M.Donike, H.Geyer, A.Gotzmann, U.Mareck-Engelke (Eds.) Recent advances in doping analysis (3) Sport und Buch Strauß, Köln (1996) 143-156.

14. Mareck-Engelke, U., Geyer, H., Donike, M.: Stability of steroid profiles (3): Ratios and excretion rates of endogenous steroids in male urines collected over 24 hours. In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995) 121-134
15. Mareck-Engelke, U., Geyer, H., Donike, M.: Stability of steroid profiles (4): Ratios and excretion rates of endogenous steroids in female urines collected four times over 24 hours. In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995) 135-156.
16. Nitschke, R.: Steroidprofile und Veränderungen biochemischer Parameter von Hochleistungsradrennfahrern unter extremen Ausdauerbelastungen während zwei Rundfahrten. Dissertation, Deutsche Sporthochschule Köln, 1997.
17. Donike, M., Rauth, S., Mareck-Engelke, U., Geyer, H., Nitschke, R.: Evaluation of longitudinal studies, the determination of subject based reference ranges of the testosterone/ epitestosterone ratio. In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth (eds.) Recent advances in doping analysis. Sport und Buch Strauß, Köln (1994) 33-40.
18. Horning, S., Schänzer, W., Donike, M.: GC/HRMS determination of the serum (plasma) testosterone/17 α -hydroxyprogesterone ratio and its significance. In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln (1995) 51-58.
19. Kicman, A.T., Oftebro, H., Walker, C., Norma, N., Cowan, D.A.: Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes. Clin. Chem. 39/9 (1993) 1798-1803.

Shah

June 21, 2001

To: Clients of the UCLA Olympic Laboratory

Regarding: **Carbon Isotope Ratio measurements, Update 3**

This letter is to update you on our carbon isotope ratio method and to explain the wording we use in our current reports.

DIOL ASSAY

Requests: Currently when you request a CIR analysis we perform the "Diol" assay. The procedure is to request a CIR analysis by fax or email and to provide the sample number. We will check the original data on the sample and determine if the analysis is likely to be successful. If not, we will advise you that we do not believe the analysis will be successful and we may suggest an alternative approach. In order to enhance our understanding of the analysis we also ask that you give us all sample numbers of prior samples from the same athlete that were analyzed at UCLA.

The "Diol" assay determines the carbon isotope ratio (delta value) of two diol metabolites of testosterone which we refer to as M1 and M2, and one metabolite of a testosterone precursor (Pdiol). [See the metabolic map attached.] The assay determines the ratio of $^{13}\text{C}/^{12}\text{C}$ for each of these three steroids. The units are usually called "delta units". In addition to the delta values for these steroids, two other types of measurements are calculated. The first is the ratio of the metabolites to the precursor. Two ratios are calculated: M1/Pdiol and M2/Pdiol. The second is the difference between the metabolites and the Pdiol: M1 Pdiol and M2-Pdiol.

Endogenous reference compound: The Pdiol serves as an endogenous reference compound (ERC). Since it is a metabolite of a precursor (see map) in the testosterone metabolic scheme, its delta value does not change when testosterone is administered. In the typical positive case, the delta values of M1 and M2 are low and the delta value of Pdiol is within the normal range. In negative cases, all three diols have similar delta values. The reporting terminology will be: Positive, Negative, or Indeterminate.

A **POSITIVE** report means that the delta values for both M1 and M2 are at least three standard deviation (SD) units less than the mean (average) of a group of 73 normal males, and the delta value for Pdiol is within 3 SD of the mean of normal males. In addition the two ratios (M1/Pdiol and M2/Pdiol) and the two differences (M1-Pdiol and M2-Pdiol) are more than 3 SD from the range of normal values. These criteria are very conservative because all must be met for the sample to be declared positive.

A **NEGATIVE** report means that all three delta values, the two difference scores, and the two ratios are within the normal range.

An **INDETERMINATE** report means that we were not able to obtain definitive data. The most common reasons for this are insufficient sample volume and low concentrations of the steroids. Since there are several criteria for a positive report, it is also possible that a sample will have one ratio and one difference score that are normal and the other ratio and difference score will be abnormal. Other combinations of results are possible. Based on our current understanding of the theory underlying CIR measurements, we believe that in these mixed cases that we classify as indeterminate, some of the molecules of M1 or M2 are derived from pharmaceutical testosterone (or another exogenous steroid which is metabolized to

testosterone) and some of the molecules are from natural (endogenous) testosterone. This is expected when the body contains a mixture of pharmaceutical and natural testosterone and metabolites. This situation is likely to occur at the later times in the curve that relates delta values to time since drug administration. As our clients gain further understanding of the CIR analysis, you may have other opinions about how we report mixed cases.

SAMPLES FROM OTHER LABORATORIES

Occasionally we are asked to perform a CIR analysis on a sample that was originally processed at another sample. We are not comfortable with this for four reasons: 1) we do not know the details of handling and storage of the sample in the other laboratory, 2) we do not have any control over the chain-of-custody until the sample arrives at UCLA, 3) we do not have screening data (T/E, testosterone concentration, etc.) obtained at UCLA therefore we cannot adequately plan the analysis, and 4) we do not have control samples that were handled in the same way. We do not have any reason to believe that the CIR analysis is affected by storage conditions, time factors, or temperature nevertheless we cannot be absolutely certain that our in-house control data applies to such samples. In the future we will decline to perform the CIR analysis unless there is sufficient sample volume to perform both a steroid analysis and a CIR analysis.

ANDRO & ETIO ASSAY

Under certain circumstances we also offer the Andro & Etio assay. The advantages are that it is simpler to perform, less expensive, and it almost always yields definitive results. The disadvantage is that there is no PdIOL to serve as an ERC, therefore we are not able to calculate ratios or difference scores. Most clients seem to prefer the DIOL assay, however we suspect that in the future the Andro&Etio assay will be developed to the point where the lack of an ERC will not be a disadvantage. At this time we recommend the "diol" assay. Occasionally we will advise you that it is not possible to perform the DIOL assay and that the only CIR option is the Andro & Etio assay.

GENDER

At the present time we do not recommend performing the CIR analysis on samples from females. If you still wish to have the analysis we will determine if it is feasible to proceed based on the existing data from the athlete.

EPITESTOSTERONE

We have been working on a CIR assay for epitestosterone and we have recently presented our findings at a national meeting. There is no peer reviewed publication at this time and it will take about a year to prepare a manuscript and get it published. Nevertheless, at your request we will conduct the epitestosterone analysis on urine samples reported "epitestosterone > 200 ng/ml".

TURN-AROUND-TIME

In an attempt to keep the costs down we are performing the analyses one week per month. Typically this is the fourth week of the month. It takes about a week to complete the analysis, calculate the results, and the issue the report.

World Anti-Doping Code



2003

TABLE OF CONTENTS

INTRODUCTION

| | |
|---|---|
| PURPOSE, SCOPE AND ORGANIZATION OF THE WORLD ANTI-DOPING PROGRAM AND THE CODE..... | 1 |
| THE WORLD ANTI-DOPING PROGRAM..... | 1 |
| THE CODE..... | 1 |
| INTERNATIONAL STANDARDS..... | 2 |
| MODELS OF BEST PRACTICE..... | 2 |
| FUNDAMENTAL RATIONALE FOR THE WORLD ANTI-DOPING CODE..... | 3 |

PART ONE: DOPING CONTROL

| | |
|---|----|
| INTRODUCTION | 6 |
| ARTICLE 1: DEFINITION OF DOPING | 8 |
| ARTICLE 2: ANTI-DOPING RULE VIOLATIONS | 8 |
| 2.1.....THE PRESENCE OF A PROHIBITED SUBSTANCE OR ITS METABOLITES OR MARKERS IN AN ATHLETE'S BODILY SPECIMEN..... | 8 |
| 2.2.....USE OR ATTEMPTED USE OF A PROHIBITED SUBSTANCE OR A PROHIBITED METHOD..... | 10 |
| 2.3..... | 10 |
| 2.4..... | 11 |
| 2.5.....TAMPERING, OR ATTEMPTING TO TAMPER, WITH ANY PART OF DOPING CONTROL..... | 11 |
| 2.6.....POSSESSION OF PROHIBITED SUBSTANCES AND METHODS..... | 11 |
| 2.7.....TRAFFICKING IN ANY PROHIBITED SUBSTANCE OR PROHIBITED METHOD..... | 12 |
| 2.8..... | 12 |
| ARTICLE 3: PROOF OF DOPING | 12 |
| 3.1.....BURDENS AND STANDARDS OF PROOF..... | 12 |
| 3.2.....METHODS OF ESTABLISHING FACTS AND PRESUMPTIONS..... | 12 |

World Anti-Doping Code
March 2003

Published by:

World Anti-Doping Agency
Stock Exchange Tower
800 Place Victoria (Suite 1700)
PO Box 120
Montreal, Quebec,
Canada H4Z 1B7

URL: www.wada-ama.org

Tel: +1.514.904.9232
Fax: +1.514.904.8650
E-mail: code@wada-ama.org

Contents

| | |
|---|-----------|
| ARTICLE 4: THE PROHIBITED LIST | 14 |
| 4.1 PUBLICATION AND REVISION OF THE PROHIBITED LIST | 14 |
| 4.2 PROHIBITED SUBSTANCES AND PROHIBITED METHODS IDENTIFIED ON THE PROHIBITED LIST | 14 |
| 4.3 CRITERIA FOR INCLUDING SUBSTANCES AND METHODS ON THE PROHIBITED LIST | 15 |
| 4.4 THERAPEUTIC USE | 17 |
| 4.5 MONITORING PROGRAM | 18 |
| ARTICLE 5: TESTING | 19 |
| 5.1 TEST DISTRIBUTION PLANNING | 19 |
| 5.2 STANDARDS FOR TESTING | 20 |
| ARTICLE 6: ANALYSIS OF SAMPLES | 20 |
| 6.1 USE OF APPROVED LABORATORIES | 20 |
| 6.2 SUBSTANCES SUBJECT TO DETECTION | 20 |
| 6.3 RESEARCH ON SAMPLES | 20 |
| 6.4 STANDARDS FOR SAMPLE ANALYSIS AND REPORTING | 21 |
| ARTICLE 7: RESULTS MANAGEMENT | 21 |
| 7.1 INITIAL REVIEW REGARDING ADVERSE ANALYTICAL FINDINGS | 21 |
| 7.2 NOTIFICATION AFTER INITIAL REVIEW | 21 |
| 7.3 FURTHER REVIEW OF ADVERSE ANALYTICAL FINDING WHERE REQUIRED BY PROHIBITED LIST | 22 |
| 7.4 REVIEW OF OTHER ANTI-DOPING RULE VIOLATIONS | 22 |
| 7.5 PRINCIPLES APPLICABLE TO PROVISIONAL SUSPENSIONS | 23 |
| ARTICLE 8: RIGHT TO A FAIR HEARING | 24 |
| ARTICLE 9: AUTOMATIC DISQUALIFICATION OF INDIVIDUAL RESULTS | 25 |
| ARTICLE 10: SANCTIONS ON INDIVIDUALS | 26 |
| 10.1 DISQUALIFICATION OF RESULTS IN EVENT DURING WHICH AN ANTI-DOPING RULE VIOLATION OCCURS | 26 |
| 10.2 IMPOSITION OF INELIGIBILITY FOR PROHIBITED SUBSTANCES AND PROHIBITED METHODS | 26 |
| 10.3 SPECIFIED SUBSTANCES | 27 |
| 10.4 INELIGIBILITY FOR OTHER ANTI-DOPING RULE VIOLATIONS | 28 |

Contents

| | |
|--|-----------|
| 10.5 ELIMINATION OR REDUCTION OF PERIOD OF INELIGIBILITY BASED ON EXCEPTIONAL CIRCUMSTANCES | 29 |
| 10.6 RULES FOR CERTAIN POTENTIAL MULTIPLE VIOLATIONS | 32 |
| 10.7 DISQUALIFICATION OF RESULTS IN COMPETITIONS SUBSEQUENT TO SAMPLE COLLECTION | 34 |
| 10.8 COMMENCEMENT OF INELIGIBILITY PERIOD | 34 |
| 10.9 STATUS DURING INELIGIBILITY | 35 |
| 10.10 REINSTATEMENT TESTING | 36 |
| ARTICLE 11: CONSEQUENCES TO TEAMS | 36 |
| ARTICLE 12: SANCTIONS AGAINST SPORTING BODIES | 37 |
| ARTICLE 13: APPEALS | 37 |
| 13.1 DECISIONS SUBJECT TO APPEAL | 37 |
| 13.2 APPEALS FROM DECISIONS REGARDING ANTI-DOPING RULE VIOLATIONS, CONSEQUENCES, AND PROVISIONAL SUSPENSIONS | 37 |
| 13.3 APPEALS FROM DECISIONS GRANTING OR DENYING A THERAPEUTIC USE EXEMPTION | 39 |
| 13.4 APPEALS FROM DECISIONS IMPOSING CONSEQUENCES UNDER PART THREE OF THE CODE | 40 |
| 13.5 APPEALS FROM DECISIONS SUSPENDING OR REVOKING LABORATORY ACCREDITATION | 40 |
| ARTICLE 14: CONFIDENTIALITY AND REPORTING | 40 |
| 14.1 INFORMATION CONCERNING ADVERSE ANALYTICAL FINDINGS AND OTHER POTENTIAL ANTI-DOPING RULE VIOLATIONS | 40 |
| 14.2 PUBLIC DISCLOSURE | 41 |
| 14.3 ATHLETE WHEREABOUTS INFORMATION | 42 |
| 14.4 STATISTICAL REPORTING | 42 |
| 14.5 DOPING CONTROL INFORMATION CLEARING HOUSE | 42 |
| ARTICLE 15: CLARIFICATION OF DOPING CONTROL RESPONSIBILITIES | 43 |
| 15.1 EVENT TESTING | 43 |
| 15.2 OUT-OF-COMPETITION TESTING | 44 |
| 15.3 RESULTS MANAGEMENT HEARINGS AND SANCTIONS | 44 |
| 15.4 MUTUAL RECOGNITION | 45 |

Contents

| | |
|--|-----------|
| ARTICLE 16: DOPING CONTROL FOR ANIMALS COMPETING IN SPORT | 45 |
| 16.1 | 45 |
| 16.2 | 46 |
| ARTICLE 17: STATUTE OF LIMITATIONS | 46 |

PART TWO: EDUCATION AND RESEARCH

| | |
|--|-----------|
| ARTICLE 18: EDUCATION | 50 |
| 18.1 BASIC PRINCIPLE AND PRIMARY GOAL | 50 |
| 18.2 PROGRAM AND ACTIVITIES | 50 |
| 18.3 COORDINATION AND COOPERATION | 50 |
| ARTICLE 19: RESEARCH | 51 |
| 19.1 PURPOSE OF ANTI-DOPING RESEARCH | 51 |
| 19.2 TYPES OF RESEARCH | 51 |
| 19.3 COORDINATION | 51 |
| 19.4 RESEARCH PRACTICES | 51 |
| 19.5 ADMINISTRATION OF PROHIBITED SUBSTANCES AND PROHIBITED METHODS | 51 |
| 19.6 MISUSE OF RESULTS | 51 |

PART THREE: ROLES AND RESPONSIBILITIES

| | |
|--|-----------|
| ARTICLE 20: ADDITIONAL ROLES AND RESPONSIBILITIES OF SIGNATORIES | 54 |
| 20.1 ROLES AND RESPONSIBILITIES OF THE INTERNATIONAL OLYMPIC COMMITTEE | 54 |
| 20.2 ROLES AND RESPONSIBILITIES OF THE INTERNATIONAL PARALYMPIC COMMITTEE | 54 |
| 20.3 ROLES AND RESPONSIBILITIES OF INTERNATIONAL FEDERATIONS | 55 |
| 20.4 ROLES AND RESPONSIBILITIES OF NATIONAL OLYMPIC COMMITTEES AND NATIONAL PARALYMPIC COMMITTEES | 56 |
| 20.5 ROLES AND RESPONSIBILITIES OF NATIONAL ANTI-DOPING ORGANIZATIONS | 57 |

Contents

| | |
|---|----|
| 20.6 ROLES AND RESPONSIBILITIES OF MAJOR EVENT ORGANIZATIONS | 57 |
| 20.7 ROLES AND RESPONSIBILITIES OF WADA | 57 |

| | |
|---|-----------|
| ARTICLE 21: ROLES AND RESPONSIBILITIES OF PARTICIPANTS | 58 |
| 21.1 ROLES AND RESPONSIBILITIES OF ATHLETES | 58 |
| 21.2 ROLES AND RESPONSIBILITIES OF ATHLETE SUPPORT PERSONNEL | 58 |

| | |
|---|-----------|
| ARTICLE 22: INVOLVEMENT OF GOVERNMENTS | 59 |
| 22.1 | 59 |
| 22.2 | 60 |
| 22.2 | 60 |

PART FOUR: ACCEPTANCE, COMPLIANCE, MODIFICATION & INTERPRETATION

| | |
|--|-----------|
| ARTICLE 23: ACCEPTANCE, COMPLIANCE AND MODIFICATION | 64 |
| 23.1 ACCEPTANCE OF THE CODE | 64 |
| 23.2 IMPLEMENTATION OF THE CODE | 64 |
| 23.3 ACCEPTANCE AND IMPLEMENTATION DEADLINES | 65 |
| 23.4 MONITORING COMPLIANCE WITH THE CODE | 65 |
| 23.5 CONSEQUENCES OF NONCOMPLIANCE WITH THE CODE | 66 |
| 23.6 MODIFICATION OF THE CODE | 66 |
| 23.7 WITHDRAWAL OF ACCEPTANCE OF THE CODE | 67 |

| | |
|---|-----------|
| ARTICLE 24: INTERPRETATION OF THE CODE | 67 |
| 24.1 | 67 |
| 24.2 | 67 |
| 24.3 | 67 |
| 24.4 | 68 |
| 24.5 | 68 |
| 24.6 | 68 |

| | |
|--------------------------------------|-----------|
| APPENDIX 1: DEFINITIONS | 72 |
|--------------------------------------|-----------|

INTRODUCTION

THE PURPOSE, SCOPE AND ORGANIZATION OF THE WORLD ANTI-DOPING PROGRAM AND THE *CODE*

The purposes of the World Anti-Doping Program and the *Code* are:

- To protect the *Athletes'* fundamental right to participate in doping-free sport and thus promote health, fairness and equality for *Athletes* worldwide; and
- To ensure harmonized, coordinated and effective anti-doping programs at the international and national level with regard to detection, deterrence and prevention of doping.

THE WORLD ANTI-DOPING PROGRAM

The World Anti-Doping Program encompasses all of the elements needed in order to ensure optimal harmonization and best practice in international and national anti-doping programs. The main elements are:

Level 1: The *Code*

Level 2: *International Standards*

Level 3: Models of Best Practice

THE *CODE*

The *Code* is the fundamental and universal document upon which the World Anti-Doping Program in sport is based. The purpose of the *Code* is to advance the anti-doping effort through universal harmonization of core anti-doping elements. It is intended to be specific enough to achieve complete harmonization on issues where uniformity is required, yet general enough in other areas to permit flexibility on how agreed upon anti-doping principles are implemented.

INTERNATIONAL STANDARDS

International Standards for different technical and operational areas within the anti-doping program will be developed in consultation with the *Signatories* and governments and approved by WADA. The purpose of the *International Standards* is harmonization among *Anti-Doping Organizations* responsible for specific technical and operational parts of the anti-doping programs. Adherence to the *International Standards* is mandatory for compliance with the *Code*. The *International Standards* may be revised from time to time by the WADA Executive Committee after reasonable consultation with the *Signatories* and governments. Unless provided otherwise in the *Code*, *International Standards* and all revisions shall become effective on the date specified in the *International Standard* or revision.

MODELS OF BEST PRACTICE

Models of Best Practice based on the *Code* will be developed to provide state of the art solutions in different areas of anti-doping. The Models will be recommended by WADA and made available to *Signatories* upon request but will not be mandatory. In addition to providing models of anti-doping documentation, WADA will also make some training assistance available to the *Signatories*.

International Standards Comment: *International Standards* will contain much of the technical detail necessary for implementing the *Code*. This would include, for example, the detailed requirements for Sample collection, laboratory analysis and laboratory accreditation currently found in the Olympic Movement Anti-Doping Code 1999 ("OMADC"). *International Standards*, while expressly incorporated into the *Code* by reference, will, in consultation with the *Signatories* and governments, be developed by experts and set forth in separate technical documents. It is

important that the technical experts be able to make timely changes to the *International Standards* without requiring any amendment of the *Code* or individual stakeholder rules and regulations.

All applicable *International Standards* will be in place by January 1, 2004.

Models of Best Practice Comment: WADA will prepare model anti-doping rules and regulations tailored to the needs of each of the major groups of *Signatories* (e.g., *International Federations* for individual sports,

FUNDAMENTAL RATIONALE FOR THE WORLD ANTI-DOPING CODE

Anti-doping programs seek to preserve what is intrinsically valuable about sport. This intrinsic value is often referred to as "the spirit of sport"; it is the essence of Olympism: it is how we play true. The spirit of sport is the celebration of the human spirit, body and mind, and is characterized by the following values:

- Ethics, fair play and honesty.
- Health.
- Excellence in performance.
- Character and education.
- Fun and joy.
- Teamwork.
- Dedication and commitment.
- Respect for rules and laws.
- Respect for self and other participants.
- Courage.
- Community and solidarity.

Doping is fundamentally contrary to the spirit of sport.

International Federations for team sports, *National Anti-Doping Organizations*, etc.). These model rules and regulations will conform with and be based on the *Code*, will be state of the art examples of best practices and will contain all of the detail (including reference to *International Standards*) necessary to conduct an effective anti-doping program.

These model rules and regulations will provide alternatives from which stakeholders may select. Some stakeholders may choose to adopt the model rules and regulations and other models of best practices verbatim. Others may decide to adopt the models with modifications. Still other stakeholders may choose to develop

their own rules and regulations consistent with the general principles and specific requirements set forth in the *Code*.

Other model documents for specific parts of the anti-doping work may be developed based on generally recognized stakeholder needs and expectations. This could include models for national anti-doping programs, results management, Testing (beyond the specific requirements set forth in the *International Standard for Testing*), education programs, etc. All Models of Best Practice will be reviewed and approved by WADA before they are included in the World Anti-Doping Program.

PART ONE

DOPING CONTROL

INTRODUCTION

Part One of the *Code* sets forth specific anti-doping rules and principles that are to be followed by organizations responsible for adopting, implementing or enforcing anti-doping rules within their authority - - e.g., the International Olympic Committee, International Paralympic Committee, International Federations, *Major Event Organizations*, and *National Anti-Doping Organizations*. All of these organizations are collectively referred to as *Anti-Doping Organizations*.

Part One of the *Code* does not replace, or eliminate the need for, comprehensive anti-doping rules adopted by each of these *Anti-Doping Organizations*. While some provisions of Part One of the *Code* must be incorporated essentially verbatim by each *Anti-Doping Organization* in its own anti-doping rules, other provisions of Part One establish mandatory guiding principles that allow flexibility in the formulation of rules by each *Anti-Doping Organization* or establish requirements that must be followed by each *Anti-Doping Organization* but need not be repeated in its own anti-doping rules. The following Articles, as applicable to the scope of anti-doping activity which the *Anti-Doping Organization* performs, must be incorporated into the rules of each *Anti-Doping Organization* without any substantive changes (allowing for necessary non-substantive editing

Introduction Comment: For example it is critical to harmonization that all Signatories base their decisions on the same list of anti-doping rule violations, the same burdens of proof and impose the same Consequences for the same anti-doping rule violations. These substantive rules must be the same whether a hearing takes place before an International Federation, at the national level or before CAS. On the other hand, it is not necessary for effective harmonization to force all Signatories to use one single results management and hearing process.

At present, there are many different, yet equally effective processes for results management and hearings within different International Federations and different national bodies. The Code does not require absolute uniformity in results management and hearing procedures; it does, however, require that the diverse approaches of the Signatories satisfy principles stated in the Code.

With respect to Article 13, subpart 13.2.2 is not included in the provisions required to be adopted essentially

changes to the language in order to refer to the organization's name, sport, section numbers, etc.): Articles 1 (Definition of Doping), 2 (Anti-Doping Rule Violations), 3 (Proof of Doping), 9 (Automatic *Disqualification* of Individual Results), 10 (Sanctions on Individuals), 11 (*Consequences* to Teams), 13 (Appeals) with the exception of 13.2.2, 17 (Statute of Limitations) and Definitions.

Anti-doping rules, like competition rules, are sport rules governing the conditions under which sport is played. *Athletes* accept these rules as a condition of participation. Anti-doping rules are not intended to be subject to or limited by the requirements and legal standards applicable to criminal proceedings or employment matters. The policies and minimum standards set forth in the *Code* represent the consensus of a broad spectrum of stakeholders with an interest in fair sport and should be respected by all courts and adjudicating bodies.

Participants shall be bound to comply with the anti-doping rules adopted in conformance with the *Code* by the relevant *Anti-Doping Organizations*. Each *Signatory* shall establish rules and procedures to ensure that all *Participants* under the authority of the *Signatory* and its member organizations are informed of and agree to be bound by anti-doping rules in force of the relevant *Anti-Doping Organizations*.

verbatim, as 13.2.2 establishes mandatory guiding principles that allow some flexibility in the formulation of rules by the Anti-Doping Organization.

Participants Comment: By their participation in sport, Athletes are bound by the competitive rules of their sport. In the same manner, Athletes and Athlete Support Personnel should be bound by anti-doping rules based on Article 2 of the Code by virtue of their agreements for membership, accreditation, or participation in sports organizations or sports events subject to the Code. Each Signatory, however, shall take the necessary steps to ensure that all Athletes and Athlete Support Personnel within its authority are bound by the relevant Anti-Doping Organization's anti-doping rules.

ARTICLE 1: DEFINITION OF DOPING

Doping is defined as the occurrence of one or more of the anti-doping rule violations set forth in Article 2.1 through Article 2.8 of the Code.

ARTICLE 2: ANTI-DOPING RULE VIOLATIONS

The following constitute anti-doping rule violations:

2.1 The presence of a *Prohibited Substance* or its *Metabolites* or *Markers* in an *Athlete's* bodily *Specimen*.

2.1.1 It is each *Athlete's* personal duty to ensure that no *Prohibited Substance* enters his or her body. *Athletes* are responsible for any *Prohibited Substance* or its *Metabolites* or *Markers* found to be present in their bodily *Specimens*. Accordingly, it is not necessary that intent, fault, negligence or knowing *Use* on the *Athlete's* part be demonstrated in order to establish an anti-doping violation under Article 2.1.

2 Comment: The purpose of Article 2 is to specify the circumstances and conduct which constitute violations of anti-doping rules. Hearings in doping cases will proceed based on the assertion that one or more of these specific rules have been violated. Most of the circumstances and conduct on this list of violations can be found in some form in the OMADC or other existing anti-doping rules.

2.1.1 Comment: For purposes of anti-doping violations involving the presence of a *Prohibited Substance* (or its *Metabolites* or *Markers*), the Code adopts the rule of strict liability which is found in the OMADC and the vast majority of existing anti-doping rules. Under the strict liability principle, an anti-doping rule violation occurs whenever a *Prohibited Substance* is found in an *Athlete's* bodily *Specimen*. The violation occurs whether

or not the *Athlete* intentionally or unintentionally used a *Prohibited Substance* or was negligent or otherwise at fault. If the positive *Sample* came from an *In-Competition* test, then the results of that *Competition* are automatically invalidated (Article 9 (Automatic Disqualification of Individual Results)). However, the *Athlete* then has the possibility to avoid or reduce sanctions if the *Athlete* can demonstrate that he or she was not at fault or significant fault (Article 10.5 (Elimination or Reduction of Period of Ineligibility Based on Exceptional Circumstances)).

The strict liability rule for the finding of a *Prohibited Substance* in an *Athlete's* *Specimen*, with a possibility that sanctions may be modified based on specified criteria, provides a reasonable balance between effective anti-doping

2.1.2 Excepting those substances for which a quantitative reporting threshold is specifically identified in the *Prohibited List*, the detected presence of any quantity of a *Prohibited Substance* or its *Metabolites* or *Markers* in an *Athlete's Sample* shall constitute an anti-doping rule violation.

2.1.3 As an exception to the general rule of Article 2.1, the *Prohibited List* may establish special criteria for the evaluation of *Prohibited Substances* that can also be produced endogenously.

enforcement for the benefit of all "clean" *Athletes* and fairness in the exceptional circumstance where a *Prohibited Substance* entered an *Athlete's* system through no fault or negligence on the *Athlete's* part. It is important to emphasize that while the determination of whether the anti-doping rule has been violated is based on strict liability, the imposition of a fixed period of ineligibility is not automatic.

The rationale for the strict liability rule was well stated by the Court of Arbitration for Sport in the case of Quigley v. UIT.

"It is true that a strict liability test is likely in some sense to be unfair in an individual case, such as that of Q., where the *Athlete* may have taken medication as the result of mislabeling or faulty advice for which he or she is not responsible - particularly in the circumstances of sudden illness in a foreign country. But it is also in some sense "unfair" for an *Athlete* to get food poisoning on the eve of an important competition. Yet in neither case will the rules of the competition be altered to undo the unfairness. Just as the competition will not be postponed to await the *Athlete's* recovery, so the prohibition of banned substances will not be lifted in recognition of its accidental absorption. The vicissitudes of competition, like those of life generally,

may create many types of unfairness whether by accident or the negligence of unaccountable Persons, which the law cannot repair.

Furthermore, it appears to be a laudable policy objective not to repair an accidental unfairness to an individual by creating an intentional unfairness to the whole body of other competitors. This is what would happen if banned performance-enhancing substances were tolerated when absorbed inadvertently. Moreover, it is likely that even intentional abuse would in many cases escape sanction for lack of proof of guilty intent. And it is certain that a requirement of intent would invite costly litigation that may well cripple federations - particularly those run on modest budgets - in their fight against doping."

2.1.3 Comment: For example, the *Prohibited List* might provide that a T/E ratio greater than 6:1 is doping unless a longitudinal analysis of prior or subsequent test results by the Anti-Doping Organization demonstrates a naturally elevated ratio or the *Athlete* otherwise establishes that the elevated ratio is the result of a physiological or pathological condition.

2.2 Use or Attempted Use of a Prohibited Substance or a Prohibited Method.

2.2.1 The success or failure of the Use of a Prohibited Substance or Prohibited Method is not material. It is sufficient that the Prohibited Substance or Prohibited Method was Used or Attempted to be Used for an anti-doping rule violation to be committed.

2.3 Refusing, or failing without compelling justification, to submit to Sample collection after notification as authorized in applicable anti-doping rules or otherwise evading Sample collection.

2.2.1 Comment: The prohibition against "Use" has been expanded from the text in the OMADC to include Prohibited Substances as well as Prohibited Methods. With this inclusion there is no need to specifically delineate "admission of Use" as a separate anti-doping rule violation. "Use" can be proved, for example, through admissions, third party testimony or other evidence.

Demonstrating the "Attempted Use" of a Prohibited Substance requires proof of intent on the Athlete's part. The fact that intent may be required to prove this particular anti-doping rule violation does not undermine the strict liability principle established for violations of Article 2.1 and Use of a Prohibited Substance or Prohibited Method.

An Athlete's Out-of-Competition Use of a Prohibited Substance that is not prohibited Out-of-Competition would not constitute an anti-doping rule violation.

2.3 Comment: Failure or refusal to submit to Sample collection after notification is prohibited in almost all existing anti-doping rules. This Article expands the typical rule to include "otherwise evading Sample collection" as prohibited conduct. Thus, for example, it would be an anti-doping rule violation if it were established that an Athlete was hiding from a Doping Control official who was attempting to conduct a test. A violation of "refusing or failing to submit to Sample collection" may be based on either intentional or negligent conduct of the Athlete, while "evading" Sample collection contemplates intentional conduct by the Athlete.

2.4 Violation of applicable requirements regarding Athlete availability for Out-of-Competition Testing including failure to provide required whereabouts information and missed tests which are declared based on reasonable rules.

2.5 Tampering, or Attempting to tamper, with any part of Doping Control.

2.6 Possession of Prohibited Substances and Methods:

2.6.1 Possession by an Athlete at any time or place of a substance that is prohibited in Out-of-Competition Testing or a Prohibited Method unless the Athlete establishes that the Possession is pursuant to a therapeutic use exemption granted in accordance with Article 4.4 (Therapeutic Use) or other acceptable justification.

2.6.2 Possession of a substance that is prohibited in Out-of-Competition Testing or a Prohibited Method by Athlete Support Personnel in connection with an Athlete, Competition or training, unless the Athlete Support Personnel establishes that the Possession is pursuant to a therapeutic use exemption granted to an Athlete in accordance with Article 4.4 (Therapeutic Use) or other acceptable justification.

2.4 Comment: Unannounced Out-of-Competition Testing is at the core of effective Doping Control. Without accurate Athlete location information such Testing is inefficient and sometimes impossible. This Article, which is not typically found in most existing anti-doping rules, requires Athletes that have been identified for Out-of-Competition Testing to be responsible for providing and updating information on their whereabouts so that they can be located for No Advance Notice Out-of-Competition Testing. The "applicable requirements" are set by the Athlete's International Federation and National

Anti-Doping Organization in order to allow some flexibility based upon varying circumstances encountered in different sports and countries. A violation of this Article may be based on either intentional or negligent conduct by the Athlete.

2.5 Comment: This Article prohibits conduct which subverts the Doping Control process but which would not be included in the typical definition of Prohibited Methods. For example, altering identification numbers on a Doping Control form during Testing or breaking the B Bottle at the time of B Sample analysis.

- 2.7** *Trafficking in any Prohibited Substance or Prohibited Method.*
- 2.8** Administration or Attempted administration of a *Prohibited Substance* or *Prohibited Method* to any *Athlete*, or assisting, encouraging, aiding, abetting, covering up or any other type of complicity involving an anti-doping rule violation or any *Attempted* violation.

ARTICLE 3: PROOF OF DOPING

3.1 Burdens and Standards of Proof.

The *Anti-Doping Organization* shall have the burden of establishing that an anti-doping rule violation has occurred. The standard of proof shall be whether the *Anti-Doping Organization* has established an anti-doping rule violation to the comfortable satisfaction of the hearing body bearing in mind the seriousness of the allegation which is made. This standard of proof in all cases is greater than a mere balance of probability but less than proof beyond a reasonable doubt. Where the *Code* places the burden of proof upon the *Athlete* or other *Person* alleged to have committed an anti-doping rule violation to rebut a presumption or establish specified facts or circumstances, the standard of proof shall be by a balance of probability.

3.2 Methods of Establishing Facts and Presumptions.

Facts related to anti-doping rule violations may be established by any reliable means, including admissions. The following rules of proof shall be applicable in doping cases:

3.1 Comment: This standard of proof required to be met by the *Anti-Doping Organization* is comparable to the standard which is applied in most countries to cases involving professional

misconduct. It has also been widely applied by courts and tribunals in doping cases. See, for example, the CAS decision in *N. J. Y. W. v. FINA*, CAS 98/208, 22 December 1998.

- 3.2.1** WADA-accredited laboratories are presumed to have conducted *Sample* analysis and custodial procedures in accordance with the *International Standard* for laboratory analysis. The *Athlete* may rebut this presumption by establishing that a departure from the *International Standard* occurred.

If the *Athlete* rebuts the preceding presumption by showing that a departure from the *International Standard* occurred, then the *Anti-Doping Organization* shall have the burden to establish that such departure did not cause the *Adverse Analytical Finding*.

- 3.2.2** Departures from the *International Standard* for *Testing* which did not cause an *Adverse Analytical Finding* or other anti-doping rule violation shall not invalidate such results. If the *Athlete* establishes that departures from the *International Standard* occurred during *Testing* then the *Anti-Doping Organization* shall have the burden to establish that such departures did not cause the *Adverse Analytical Finding* or the factual basis for the anti-doping rule violation.

3.2.1 Comment: The burden is on the *Athlete* to establish, by a preponderance of the evidence, a departure from the *International Standard*. If the *Athlete* does so, the

burden shifts to the *Anti-Doping Organization* to prove to the comfortable satisfaction of the hearing body that the departure did not change the test result.

ARTICLE 4: THE PROHIBITED LIST

4.1 Publication and Revision of the *Prohibited List*.

WADA shall, as often as necessary and no less often than annually, publish the *Prohibited List* as an *International Standard*. The proposed content of the *Prohibited List* and all revisions shall be provided in writing promptly to all *Signatories* and governments for comment and consultation. Each annual version of the *Prohibited List* and all revisions shall be distributed promptly by WADA to each *Signatory* and government and shall be published on WADA's website, and each *Signatory* shall take appropriate steps to distribute the *Prohibited List* to its members and constituents. The rules of each *Anti-Doping Organization* shall specify that, unless provided otherwise in the *Prohibited List* or a revision, the *Prohibited List* and revisions shall go into effect under the *Anti-Doping Organization's* rules three months after publication of the *Prohibited List* by WADA without requiring any further action by the *Anti-Doping Organization*.

4.2 *Prohibited Substances and Prohibited Methods* Identified on the *Prohibited List*.

The *Prohibited List* shall identify those *Prohibited Substances and Prohibited Methods* which are prohibited as doping at all times (both *In-Competition* and *Out-of-*

4.1 Comment: The *Prohibited List* will be revised and published on an expedited basis whenever the need arises. However, for the sake of predictability, a new list will be published every year whether or not changes have been made. The virtue of the IOC practice of publishing a new list every January is that it avoids confusion over which list is the most current. To address this issue, WADA will always have the most current *Prohibited List* published on its website.

It is anticipated that revised anti-doping rules adopted by *Anti-Doping Organizations* pursuant to the Code will not go into effect until January 1, 2004 with the publication of the first *Prohibited List* adopted by WADA. The OMADC will continue to be applicable until the Code is accepted by the International Olympic Committee.

4.2 Comment: There will be one *Prohibited List*. The substances which are prohibited at all times

Competition) because of their potential to enhance performance in future *Competitions* or their masking potential and those substances and methods which are prohibited *In-Competition* only. Upon the recommendation of an International Federation, the *Prohibited List* may be expanded by WADA for that particular sport. *Prohibited Substances and Prohibited Methods* may be included in the *Prohibited List* by general category (e.g., anabolic agents) or by specific reference to a particular substance or method.

4.3 Criteria for Including Substances and Methods on the *Prohibited List*.

WADA shall consider the following criteria in deciding whether to include a substance or method on the *Prohibited List*.

4.3.1 A substance or method shall be considered for inclusion on the *Prohibited List* if WADA determines that the substance or method meets any two of the following three criteria:

4.3.1.1 Medical or other scientific evidence, pharmacological effect or experience that the substance or method has the potential to enhance or enhances sport performance;

would include masking agents and those substances which, when used in training, may have long term performance enhancing effects such as anabolics. All substances and methods on the *Prohibited List* are prohibited *In-Competition*. This distinction between what is tested for *In-Competition* and what is tested for *Out-of-Competition* is carried over from the OMADC. There will be only one document called the "*Prohibited List*." WADA may add additional substances or methods to the *Prohibited List* for particular sports (e.g. the inclusion of beta-blockers for

shooting) but this will also be reflected on the single *Prohibited List*. Having all *Prohibited Substances* on a single list will avoid some of the current confusion related to identifying which substances are prohibited in which sports. Individual sports are not permitted to seek exemption from the basic list of *Prohibited Substances* (e.g. eliminating anabolics from the *Prohibited List* for "mind sports"). The premise of this decision is that there are certain basic doping agents which anyone who chooses to call himself or herself an Athlete should not take.

4.3.1.2 Medical or other scientific evidence, pharmacological effect, or experience that the *Use* of the substance or method represents an actual or potential health risk to the *Athlete*;

4.3.1.3 WADA's determination that the *Use* of the substance or method violates the spirit of sport described in the Introduction to the *Code*.

4.3.2 A substance or method shall also be included on the *Prohibited List* if WADA determines there is medical or other scientific evidence, pharmacological effect or experience that the substance or method has the potential to mask the *Use* of other *Prohibited Substances* and *Prohibited Methods*.

4.3.2 Comment: A substance shall be considered for inclusion on the *Prohibited List* if the substance is a masking agent or meets two of the following three criteria: (1) it has the potential to enhance or enhances sport performance; (2) it represents a potential or actual health risk; or (3) it is contrary to the spirit of sport. None of the three criteria *alone* is a sufficient basis for adding a substance to the *Prohibited List*. Using the potential to enhance performance as the sole criteria would include, for example, physical and mental training, red meat, carbohydrate loading and training at

altitude. Risk of harm would include smoking. Requiring all three criteria would also be unsatisfactory. For example the use of genetic transfer technology to dramatically enhance sport performance should be prohibited as contrary to the spirit of sport even if it is not harmful. Similarly, the potentially unhealthy abuse of certain substances without therapeutic justification based on the mistaken belief they enhance performance is certainly contrary to the spirit of sport regardless of whether the expectation of performance enhancement is realistic.

4.3.3 WADA's determination of the *Prohibited Substances* and *Prohibited Methods* that will be included on the *Prohibited List* shall be final and shall not be subject to challenge by an *Athlete* or other *Person* based on an argument that the substance or method was not a masking agent or did not have the potential to enhance performance, represent a health risk, or violate the spirit of sport.

4.4 Therapeutic Use

WADA shall adopt an *International Standard* for the process of granting therapeutic use exemptions.

Each International Federation shall ensure, for *International-Level Athletes* or any other *Athlete* who is

4.3.3 Comment: The question of whether a substance meets the criteria in Article 4.3 (Criteria for Including Substances and Methods on the *Prohibited List*) in a particular case cannot be raised as a defense to an anti-doping rule violation. For example, it cannot be argued that the *Prohibited Substance* detected would not have been performance enhancing in that particular sport. Rather, doping occurs when a substance on the *Prohibited List* is found in an *Athlete's* bodily Specimen. The same principle is found in the OMADC.

4.4 Comment: It is important that the processes for granting therapeutic use exemptions become more harmonized. *Athletes* who use medically prescribed *Prohibited Substances* may be subject to sanctioning unless they have previously obtained a therapeutic use exemption. However, currently many sporting bodies have no rules permitting therapeutic use exemptions; others follow unwritten policies; and only a few have written policies incorporated into their anti-doping rules. This Article seeks to harmonize the basis upon which therapeutic use exemptions will be

granted and gives responsibility for granting or denying exemptions to the International Federations for *International-Level Athletes* and to the National Anti-Doping Organizations for national-level *Athletes* (that are not also *International-Level Athletes*) and other *Athletes* subject to Doping Control under the *Code*.

Examples of commonly prescribed *Prohibited Substances* which might be specifically addressed in the *International Standard* for therapeutic use exemptions are medications prescribed for acute severe asthma and inflammatory bowel disease. When a therapeutic use exemption has been denied or granted in contravention of the *International Standard*, that decision may be submitted to WADA for review as provided in the *International Standard* and thereafter appealed as provided in Article 13.3 (Appeals). If the granting of a therapeutic use exemption is reversed, the reversal shall not apply retroactively and shall not disqualify the *Athlete's* results during the time that the therapeutic use exemption was in effect.

entered in an *International Event*, that a process is in place whereby *Athletes* with documented medical conditions requiring the *Use* of a *Prohibited Substance* or a *Prohibited Method* may request a therapeutic use exemption. Each *National Anti-Doping Organization* shall ensure, for all *Athletes* within its jurisdiction that are not *International-Level Athletes*, that a process is in place whereby *Athletes* with documented medical conditions requiring the *Use* of a *Prohibited Substance* or a *Prohibited Method* may request a therapeutic use exemption. Such requests shall be evaluated in accordance with the *International Standard* on therapeutic use. International Federations and *National Anti-Doping Organizations* shall promptly report to WADA the granting of therapeutic use exemptions to any *International-Level Athlete* or national-level *Athlete* that is included in his or her *National Anti-Doping Organization's Registered Testing Pool*.

WADA, on its own initiative, may review the granting of a therapeutic use exemption to any *International-Level Athlete* or national-level *Athlete* that is included in his or her *National Anti-Doping Organization's Registered Testing Pool*. Further, upon the request of any such *Athlete* that has been denied a therapeutic use exemption, WADA may review such denial. If WADA determines that such granting or denial of a therapeutic use exemption did not comply with the *International Standard* for therapeutic use exemptions, WADA may reverse the decision.

4.5 Monitoring Program

WADA, in consultation with other *Signatories* and governments, shall establish a monitoring program regarding substances which are not on the *Prohibited List*, but which WADA wishes to monitor in order to detect patterns of misuse in sport. WADA shall publish, in advance of any *Testing*, the substances that will be monitored. Laboratories will report the instances of reported *Use* or detected presence of these substances to

WADA periodically on an aggregate basis by sport and whether the *Samples* were collected *In-Competition* or *Out-of-Competition*. Such reports shall not contain additional information regarding specific *Samples*. WADA shall make available to International Federations and *National Anti-Doping Organizations*, on at least an annual basis, aggregate statistical information by sport regarding the additional substances. WADA shall implement measures to ensure that strict anonymity of individual *Athletes* is maintained with respect to such reports. The reported use or detected presence of the monitored substances shall not constitute a doping violation.

ARTICLE 5: TESTING

5.1 Test Distribution Planning. *Anti-Doping Organizations* conducting *Testing* shall in coordination with other *Anti-Doping Organizations* conducting *Testing* on the same *Athlete* pool:

5.1.1 Plan and implement an effective number of *In-Competition* and *Out-of-Competition* tests. Each International Federation shall establish a *Registered Testing Pool* for *International-Level Athletes* in its sport, and each *National Anti-Doping Organization* shall establish a national *Registered Testing Pool* for *Athletes* in its country. The national-level pool shall include *International-Level Athletes* from that country as well as other national-level *Athletes*. Each International Federation and *National Anti-Doping Organization* shall plan and conduct *In-Competition* and *Out-of-Competition Testing* on its *Registered Testing Pool*.

5.1.2 Make *No Advance Notice Testing* a priority.

5.1.3 Conduct *Target Testing*.

5.1.3 Comment: *Target Testing* is specified because random *Testing*, or even weighted random *Testing*, does

not ensure that all of the appropriate *Athletes* will be tested. (For example, world class *Athletes*, *Athletes* whose

5.2 Standards for Testing

Anti-Doping Organizations conducting *Testing* shall conduct such *Testing* in conformity with the *International Standard for Testing*.

ARTICLE 6: ANALYSIS OF SAMPLES

Doping Control Samples shall be analyzed in accordance with the following principles:

6.1 Use of Approved Laboratories

Doping Control Samples shall be analyzed only in WADA-accredited laboratories or as otherwise approved by WADA. The choice of the WADA-accredited laboratory (or other method approved by WADA) used for the *Sample* analysis shall be determined exclusively by the *Anti-Doping Organization* responsible for results management.

6.2 Substances Subject to Detection

Doping Control Samples shall be analyzed to detect *Prohibited Substances* and *Prohibited Methods* identified on the *Prohibited List* and other substances as may be directed by WADA pursuant to Article 4.5 (Monitoring Program).

6.3 Research on Samples

No *Sample* may be used for any purpose other than the detection of substances (or classes of substances) or methods on the *Prohibited List*, or as otherwise identified

performances have dramatically improved over a short period of time. Athletes whose coaches have had other Athletes test positive, etc.).

Obviously, Target Testing must not be used for any purpose other than legitimate Doping Control. The Code makes it clear that Athletes have no right to expect that they will be tested only on a random basis. Similarly, it does not impose any reasonable suspicion or probable cause requirement for Target Testing.

5.2 Comment: The required methods and processes for the various types of In-Competition and Out-of-Competition Testing will be described in greater detail in the *International Standard for Testing*.

6.1 Comment: The phrase "or other method approved by WADA" is intended to cover, for example, mobile blood Testing procedures which WADA has reviewed and considers to be reliable.

by WADA pursuant to Article 4.5 (Monitoring Program), without the *Athlete's* written consent.

6.4 Standards for Sample Analysis and Reporting

Laboratories shall analyze *Doping Control Samples* and report results in conformity with the *International Standard* for laboratory analysis.

ARTICLE 7: RESULTS MANAGEMENT

Each *Anti-Doping Organization* conducting results management shall establish a process for the pre-hearing administration of potential anti-doping rule violations that respects the following principles:

7.1 Initial Review Regarding Adverse Analytical Findings

Upon receipt of an *A Sample Adverse Analytical Finding*, the *Anti-Doping Organization* responsible for results management shall conduct a review to determine whether: (a) an applicable therapeutic use exemption has been granted, or (b) there is any apparent departure from the *International Standards for Testing* or laboratory analysis that undermines the validity of the *Adverse Analytical Finding*.

7.2 Notification After Initial Review

If the initial review under Article 7.1 does not reveal an applicable therapeutic use exemption or departure that undermines the validity of the *Adverse Analytical Finding*,

7 Comment: Various of the Signatories have created their own approaches to results management for Adverse Analytical Findings. While the various approaches have not been entirely uniform, many have proven to be fair and effective systems for results management. The Code does not supplant each of the Signatories' results management systems. This Article does, however, specify basic principles in order to ensure the fundamental

fairness of the results management process which must be observed by each Signatory. The specific anti-doping rules of each Signatory shall be consistent with these basic principles.

7.2 Comment: The Athlete has a right to request a prompt B Sample analysis regardless of whether follow-up investigation may be required under Articles 7.3 or 7.4.

the *Anti-Doping Organization* shall promptly notify the *Athlete*, in the manner set out in its rules, of: (a) the *Adverse Analytical Finding*; (b) the anti-doping rule violated, or, in a case under Article 7.3, a description of the additional investigation that will be conducted as to whether there is an anti-doping rule violation; (c) the *Athlete's* right to promptly request the analysis of the B *Sample* or, failing such request, that the B *Sample* analysis may be deemed waived; (d) the right of the *Athlete* and/or the *Athlete's* representative to attend the B *Sample* opening and analysis if such analysis is requested; and (e) the *Athlete's* right to request copies of the A and B *Sample* laboratory documentation package which includes information as required by the *International Standard* for laboratory analysis.

7.3 Further Review of Adverse Analytical Finding Where Required by Prohibited List

The *Anti-Doping Organization* or other reviewing body established by such organization shall also conduct any follow-up investigation as may be required by the *Prohibited List*. Upon completion of such follow-up investigation, the *Anti-Doping Organization* shall promptly notify the *Athlete* regarding the results of the follow-up investigation and whether or not the *Anti-Doping Organization* asserts that an anti-doping rule was violated.

7.4 Review of Other Anti-Doping Rule Violations

The *Anti-Doping Organization* or other reviewing body established by such organization shall conduct any follow-up investigation as may be required under applicable anti-doping policies and rules adopted pursuant to the *Code* or which the *Anti-Doping Organization* otherwise considers appropriate. The *Anti-Doping Organization* shall promptly give the *Athlete* or other *Person* subject to sanction notice,

7.4 Comment: As an example, an *International Federation* typically

would notify the *Athlete* through the *Athlete's* national sports federation.

in the manner set out in its rules, of the anti-doping rule which appears to have been violated, and the basis of the violation.

7.5 Principles Applicable to Provisional Suspensions

A *Signatory* may adopt rules, applicable to any *Event* for which the *Signatory* is the ruling body or for any team selection process for which the *Signatory* is responsible, permitting *Provisional Suspensions* to be imposed after the review and notification described in Articles 7.1 and 7.2 but prior to a final hearing as described in Article 8 (Right to a Fair Hearing). Provided, however, that a *Provisional Suspension* may not be imposed unless the *Athlete* is given either: (a) an opportunity for a *Provisional Hearing* either before imposition of the *Provisional Suspension* or on a timely basis after imposition of the *Provisional Suspension*; or (b) an opportunity for an expedited hearing in accordance with Article 8 (Right to a Fair Hearing) on a timely basis after imposition of a *Provisional Suspension*.

If a *Provisional Suspension* is imposed based on an A *Sample Adverse Analytical Finding* and a subsequent B *Sample* analysis does not confirm the A *Sample* analysis, then the *Athlete* shall not be subject to any further disciplinary action and any sanction previously imposed shall be rescinded. In circumstances where the *Athlete* or the *Athlete's* team has been removed from a *Competition* and the subsequent B *Sample* analysis does not confirm the A *Sample* finding, if, without otherwise affecting the *Competition*, it is still possible for the *Athlete* or team to be reinserted, the *Athlete* or team may continue to take part in the *Competition*.

7.5 Comment: This Article continues to permit the possibility of a *Provisional Suspension* before a final decision at a hearing under Article 8 (Right to a Fair Hearing). *Provisional Suspensions* have been authorized in the OMADC and by the rules of many *International Federations*. However,

before a *Provisional Suspension* can be unilaterally imposed by an *Anti-Doping Organization*, the internal review specified in the *Code* must first be completed. In addition, a *Signatory* imposing a *Provisional Suspension* is required to give the *Athlete* an opportunity for a *Provisional Hearing*

ARTICLE 8: RIGHT TO A FAIR HEARING

Each *Anti-Doping Organization* with responsibility for results management shall provide a hearing process for any *Person* who is asserted to have committed an anti-doping rule violation. Such hearing process shall address whether an anti-doping violation was committed and, if so, the appropriate *Consequences*. The hearing process shall respect the following principles:

- a timely hearing;
- fair and impartial hearing body;
- the right to be represented by counsel at the *Person's* own expense;
- the right to be fairly and timely informed of the asserted anti-doping rule violation;
- the right to respond to the asserted anti-doping rule violation and resulting *Consequences*;

either before or promptly after the imposition of the Provisional Suspension, or an expedited final hearing under Article 8 promptly after imposition of the Provisional Suspension. The Athlete has a right to appeal under Article 13.2. As an alternative to the process for imposing a Provisional Suspension under this Article, the Anti-Doping Organization may always elect to forego a Provisional Suspension and proceed directly to the final hearing utilizing an expedited process under Article 8.

In the rare circumstance where the B Sample analysis does not confirm the A Sample finding, the Athlete that had been provisionally suspended will be

allowed, where circumstances permit, to participate in subsequent Competitions during the Event. Similarly, depending upon the relevant rules of the International Federation in a Team Sport, if the team is still in Competition, the Athlete may be able to take part in future Competitions.

8 Comment: This Article contains basic principles relative to ensuring a fair hearing for Persons asserted to have violated anti-doping rules. This Article is not intended to supplant each Signatory's own rules for hearings but rather to ensure that each Signatory provides a hearing process consistent with these principles.

- the right of each party to present evidence, including the right to call and question witnesses (subject to the hearing body's discretion to accept testimony by telephone or written submission);
- the *Person's* right to an interpreter at the hearing, with the hearing body to determine the identity, and responsibility for the cost, of the interpreter; and
- a timely, written, reasoned decision;

Hearings held in connection with *Events* may be conducted by an expedited process as permitted by the rules of the relevant *Anti-Doping Organization* and the hearing body.

ARTICLE 9: AUTOMATIC DISQUALIFICATION OF INDIVIDUAL RESULTS

An anti-doping rule violation in connection with an *In-Competition* test automatically leads to *Disqualification* of the individual result obtained in that *Competition* with all resulting consequences, including forfeiture of any medals, points and prizes.

The reference to CAS as an appellate body in Article 13 does not prevent a Signatory from also specifying CAS as the initial hearing body.

For example a hearing could be expedited on the eve of a major Event where the resolution of the anti-doping rule violation is necessary to determine the Athlete's eligibility to participate in the Event or during an Event where the resolution of the case will affect the validity of the Athlete's results or continued participation in the Event.

9 Comment: This principle is found in the OMADC. When an Athlete wins a gold medal with a Prohibited Substance in his or her system, that is unfair to the other Athletes in that Competition regardless of whether the gold medalist was at fault in any way. Only a "clean" Athlete should be allowed to benefit from his or her competitive results.

For Team Sports, see Article 11 (Consequences to Teams).

ARTICLE 10: SANCTIONS ON INDIVIDUALS

10.1 *Disqualification of Results in Event During which an Anti-Doping Rule Violation Occurs*

An anti-doping rule violation occurring during or in connection with an *Event* may, upon the decision of the ruling body of the *Event*, lead to *Disqualification* of all of the *Athlete's* individual results obtained in that *Event* with all consequences, including forfeiture of all medals, points and prizes, except as provided in Article 10.1.1.

10.1.1 If the *Athlete* establishes that he or she bears *No Fault or Negligence* for the violation, the *Athlete's* individual results in the other *Competitions* shall not be *Disqualified* unless the *Athlete's* results in *Competitions* other than the *Competition* in which the anti-doping rule violation occurred were likely to have been affected by the *Athlete's* anti-doping rule violation.

10.2 *Imposition of Ineligibility for Prohibited Substances and Prohibited Methods*

Except for the specified substances identified in Article 10.3, the period of *Ineligibility* imposed for a violation of Articles 2.1 (presence of *Prohibited Substance* or its *Metabolites* or

10.1 Comment: Whereas Article 9 (Automatic Disqualification of Individual Results) Disqualifies the result in a single Competition in which the Athlete tested positive (e.g., the 100 meter backstroke), this Article may lead to Disqualification of all results in all races during the Event (e.g., the FINA World Championships).

Factors to be included in considering whether to Disqualify other results in an Event might include, for example, the severity of the Athlete's anti-doping rule violation and whether the Athlete tested negative in the other Competitions.

10.2 Comment: Harmonization of sanctions has been one of the most discussed and debated areas of anti-doping. Arguments against requiring harmonization of sanctions are based on differences between sports including for example the following: in some sports the Athletes are professionals making a sizable income from the sport and in others the Athletes are true amateurs; in those sports where an Athlete's career is short (e.g. artistic gymnastics) a two year Disqualification has a much more significant effect on the Athlete than in sports where careers are traditionally

Markers). 2.2 (Use or Attempted Use of Prohibited Substance or Prohibited Method) and 2.6 (Possession of Prohibited Substances and Methods) shall be:

- First violation: Two (2) years' *Ineligibility*.
- Second violation: Lifetime *Ineligibility*.

However, the *Athlete* or other *Person* shall have the opportunity in each case, before a period of *Ineligibility* is imposed, to establish the basis for eliminating or reducing this sanction as provided in Article 10.5

10.3 *Specified Substances*

The *Prohibited List* may identify specified substances which are particularly susceptible to unintentional anti-doping rules violations because of their general availability in medicinal products or which are less likely to be successfully abused as doping agents. Where an *Athlete* can establish that the *Use* of such a specified

much longer (e.g. equestrian and shooting); in individual sports, the Athlete is better able to maintain competitive skills through solitary practice during Disqualification than in other sports where practice as part of a team is more important. A primary argument in favor of harmonization is that it is simply not right that two Athletes from the same country who test positive for the same Prohibited Substance under similar circumstances should receive different sanctions only because they participate in different sports. In addition, flexibility in sanctioning has often been viewed as an unacceptable opportunity for some sporting bodies to be more lenient with dopers. The lack of harmonization of sanctions has also frequently been the source of jurisdictional conflicts between

International Federations and National Anti-Doping Organizations.

The consensus of the World Conference on Doping in Sport held in Lausanne in February 1999 supported a two year period of Ineligibility for a first serious anti-doping rule violation followed with a lifetime ban for a second violation. This consensus was reflected in the OMADC.

10.3 Comment: This principle is carried over from the OMADC and allows, for example, some flexibility in disciplining Athletes who test positive as a result of the inadvertent use of a cold medicine containing a prohibited stimulant. "Reduction" of a sanction under Article 10.5.2 applies only to a second or third violation because the sanction for a first

substance was not intended to enhance sport performance, the period of *Ineligibility* found in Article 10.2 shall be replaced with the following:

- *First violation:* At a minimum, a warning and reprimand and no period of *Ineligibility* from future *Events*, and at a maximum, one (1) year's *Ineligibility*.
- *Second violation:* Two (2) years' *Ineligibility*.
- *Third violation:* Lifetime *Ineligibility*.

However, the *Athlete* or other *Person* shall have the opportunity in each case, before a period of *Ineligibility* is imposed, to establish the basis for eliminating or reducing (in the case of a second or third violation) this sanction as provided in Article 10.5.

10.4 *Ineligibility* for Other Anti-Doping Rule Violations

The period of *Ineligibility* for other anti-doping rule violations shall be:

10.4.1 For violations of Article 2.3 (refusing or failing to submit to *Sample* collection) or Article 2.5 (*Tampering with Doping Control*), the *Ineligibility* periods set forth in Article 10.2 shall apply.

10.4.2 For violations of Articles 2.7 (*Trafficking*) or 2.8 (administration of *Prohibited Substance* or *Prohibited Method*), the period of *Ineligibility* imposed shall be a minimum of four (4) years up to

violation already builds in sufficient discretion to allow consideration of the Person's degree of fault.

10.4.2 Comment: Those who are involved in doping *Athletes* or covering up doping should be subject to sanctions which are more severe than the *Athletes* who test positive.

Since the authority of sport organizations is generally limited to Ineligibility for credentials, membership and other sport benefits, reporting Athlete Support Personnel to competent authorities is an important step in the deterrence of doping.

lifetime *Ineligibility*. An anti-doping rule violation involving a *Minor* shall be considered a particularly serious violation, and, if committed by *Athlete Support Personnel* for violations other than specified substances referenced in Article 10.3, shall result in lifetime *Ineligibility* for such *Athlete Support Personnel*. In addition, violations of such Articles which also violate non-sporting laws and regulations, may be reported to the competent administrative, professional or judicial authorities.

10.4.3 For violations of Article 2.4 (whereabouts violation or missed test), the period of *Ineligibility* shall be at a minimum 3 months and at a maximum 2 years in accordance with the rules established by the *Anti-Doping Organization* whose test was missed or whereabouts requirement was violated. The period of *Ineligibility* for subsequent violations of Article 2.4 shall be as established in the rules of the *Anti-Doping Organization* whose test was missed or whereabouts requirement was violated.

10.5 Elimination or Reduction of Period of *Ineligibility* Based on Exceptional Circumstances.

10.5.1 *No Fault or Negligence*

If the *Athlete* establishes in an individual case involving an anti-doping rule violation under Article

10.4.3 Comment: The whereabouts and missed test policies of different *Anti-Doping Organizations* may vary considerably, particularly at the outset as these policies are being put into place. Thus, considerable flexibility has been provided for sanctioning these anti-doping rule violations. Those *Anti-Doping Organizations* with more sophisticated policies including built in safeguards, and those organizations with longer track

records of *Athlete* experience with a whereabouts policy, could provide for *Ineligibility* periods at the longer end of the specified range

10.5.1 Comment: Article 10.5.1 applies only to violations under Articles 2.1 and 2.2 (presence and Use of Prohibited Substances) because fault or negligence is already required to establish an anti-doping rule violation under other anti-doping rules.

2.1 (presence of *Prohibited Substance* or its *Metabolites* or *Markers*) or *Use of a Prohibited Substance* or *Prohibited Method* under Article 2.2 that he or she bears *No Fault or Negligence* for the violation, the otherwise applicable period of *Ineligibility* shall be eliminated. When a *Prohibited Substance* or its *Markers* or *Metabolites* is detected in an Athlete's Specimen in violation of Article 2.1 (presence of *Prohibited Substance*), the Athlete must also establish how the *Prohibited Substance* entered his or her system in order to have the period of *Ineligibility* eliminated. In the event this Article is applied and the period of *Ineligibility* otherwise applicable is eliminated, the anti-doping rule violation shall not be considered a violation for the limited purpose of determining the period of *Ineligibility* for multiple violations under Articles 10.2, 10.3 and 10.6.

10.5.2 No Significant Fault or Negligence

This Article 10.5.2 applies only to anti-doping rule violations involving Article 2.1 (presence of *Prohibited Substance* or its *Metabolites* or *Markers*), *Use of a Prohibited Substance* or

10.5.2 Comment: The trend in doping cases has been to recognize that there must be some opportunity in the course of the hearing process to consider the unique facts and circumstances of each particular case in imposing sanctions. This principle was accepted at the World Conference on Doping in Sport 1999 and was incorporated into the OMADC which provides that sanctions can be reduced in "exceptional circumstances." The Code also provides for the possible reduction or elimination of the period of *Ineligibility* in the unique circumstance where the Athlete can establish that he or she

had *No Fault or Negligence*, or *No Significant Fault or Negligence*, in connection with the violation. This approach is consistent with basic principles of human rights and provides a balance between those Anti-Doping Organizations that argue for a much narrower exception, or none at all, and those that would reduce a two year suspension based on a range of other factors even when the Athlete was admittedly at fault. These Articles apply only to the imposition of sanctions; they are not applicable to the determination of whether an anti-doping rule violation has occurred.

Prohibited Method under Article 2.2, failing to submit to *Sample* collection under Article 2.3, or administration of a *Prohibited Substance* or *Prohibited Method* under Article 2.8. If an Athlete establishes in an individual case involving such violations that he or she bears *No Significant Fault or Negligence*, then the period of *Ineligibility* may be reduced, but the reduced period of *Ineligibility* may not be less than one-half of the minimum period of *Ineligibility* otherwise applicable. If the otherwise applicable period of *Ineligibility* is a lifetime, the reduced period under this section may be no less than 8 years. When a *Prohibited Substance* or its *Markers* or *Metabolites* is detected in an Athlete's Specimen in violation of Article 2.1 (presence of *Prohibited Substance*), the Athlete must also establish how the *Prohibited Substance* entered his or her system in order to have the period of *Ineligibility* reduced.

Article 10.5 is meant to have an impact only in cases where the circumstances are truly exceptional and not in the vast majority of cases.

To illustrate the operation of Article 10.5, an example where *No Fault or Negligence* would result in the total elimination of a sanction is where an Athlete could prove that, despite all due care, he or she was sabotaged by a competitor. Conversely, a sanction could not be completely eliminated on the basis of *No Fault or Negligence* in the following circumstances: (a) a positive test resulting from a mislabeled or contaminated vitamin or nutritional supplement (Athletes are responsible for what they ingest (Article 2.1.1) and have been warned against the possibility of supplement contamination); (b) the administration of a prohibited substance by the Athlete's personal physician or

trainer without disclosure to the Athlete (Athletes are responsible for their choice of medical personnel and for advising medical personnel that they cannot be given any prohibited substance); and (c) sabotage of the Athlete's food or drink by a spouse, coach or other person within the Athlete's circle of associates (Athletes are responsible for what they ingest and for the conduct of those persons to whom they entrust access to their food and drink). However, depending on the unique facts of a particular case, any of the referenced illustrations could result in a reduced sanction based on *No Significant Fault or Negligence*. (For example, reduction may well be appropriate in illustration (a) if the Athlete clearly establishes that the cause of the positive test was contamination in a common multiple vitamin purchased from a source with no connection to

10.5.3 Athlete's Substantial Assistance in Discovering or Establishing Anti-Doping Rule Violations by Athlete Support Personnel and Others.

An *Anti-Doping Organization* may also reduce the period of *Ineligibility* in an individual case where the *Athlete* has provided substantial assistance to the *Anti-Doping Organization* which results in the *Anti-Doping Organization* discovering or establishing an anti-doping rule violation by another *Person* involving *Possession* under Article 2.6.2 (*Possession by Athlete Support Personnel*), Article 2.7 (*Trafficking*), or Article 2.8 (administration to an *Athlete*). The reduced period of *Ineligibility* may not, however, be less than one-half of the minimum period of *Ineligibility* otherwise applicable. If the otherwise applicable period of *Ineligibility* is a lifetime, the reduced period under this section may be no less than 8 years.

10.6 Rules for Certain Potential Multiple Violations

10.6.1 For purposes of imposing sanctions under Articles 10.2, 10.3 and 10.4, a second anti-doping rule violation may be considered for purposes of imposing sanctions only if the *Anti-Doping*

Prohibited Substances and the Athlete exercised care in not taking other nutritional supplements.)

Article 10.5.2 applies only to the identified anti-doping rule violations because these violations may be based on conduct that is not intentional or purposeful. Violations under Article 2.4 (whereabouts information and missed tests) are not included, even though intentional conduct is not required to establish these violations because the sanction for violations of Article 2.4

(from three months to two years) already builds in sufficient discretion to allow consideration of the *Athlete's* degree of fault.

10.6.1 Comment: Under this Article, an *Athlete* testing positive a second time before notice of the first positive test would only be sanctioned on the basis of a single anti-doping rule violation.

Organization can establish that the *Athlete* or other *Person* committed the second anti-doping rule violation after the *Athlete* or other *Person* received notice, or after the *Anti-Doping Organization* made a reasonable *Attempt* to give notice, of the first anti-doping rule violation; if the *Anti-Doping Organization* cannot establish this, the violations shall be considered as one single first violation, and the sanction imposed shall be based on the violation that carries the more severe sanction.

10.6.2 Where an *Athlete*, based on the same *Doping Control*, is found to have committed an anti-doping rule violation involving both a specified substance under Article 10.3 and another *Prohibited Substance* or *Prohibited Method*, the *Athlete* shall be considered to have committed a single anti-doping rule violation, but the sanction imposed shall be based on the *Prohibited Substance* or *Prohibited Method* that carries the most severe sanction.

10.6.3 Where an *Athlete* is found to have committed two separate anti-doping rule violations, one involving a specified substance governed by the sanctions set forth in Article 10.3 (Specified Substances) and the

10.6.3 Comment: Article 10.6.3 deals with the situation where an *Athlete* commits two separate anti-doping rule violations, but one of the violations involves a specified substance governed by the lesser sanctions of Article 10.3. Without this Article in the Code, the second offense arguably could be governed by the sanction applicable to a second violation for the *Prohibited Substance* involved in the second violation, the sanction applicable to a second offense for the substance involved in the first violation, or a combination of the sanctions applicable to the two

offenses. This Article imposes a combined sanction calculated by adding together the sanctions for a first offense under 10.2 (two years) and a first offense under 10.3 (up to one year). This provides the same sanction to the *Athlete* that commits a first violation under 10.2 followed by a second violation involving a specified substance, and the *Athlete* that commits a first violation involving a specified substance followed by a second violation under 10.2. In both cases, the sanction shall be from two years to three years' *Ineligibility*.

other involving a *Prohibited Substance* or *Prohibited Method* governed by the sanctions set forth in Article 10.2 or a violation governed by the sanctions in Article 10.4.1, the period of *Ineligibility* imposed for the second offense shall be at a minimum two years' *Ineligibility* and at a maximum three years' *Ineligibility*. Any *Athlete* found to have committed a third anti-doping rule violation involving any combination of specified substances under Article 10.3 and any other anti-doping rule violation under 10.2 or 10.4.1 shall receive a sanction of lifetime *Ineligibility*.

10.7 Disqualification of Results in Competitions Subsequent to Sample Collection

In addition to the automatic *Disqualification* of the results in the *Competition* which produced the positive *Sample* under Article 9 (Automatic *Disqualification* of Individual Results), all other competitive results obtained from the date a positive *Sample* was collected (whether *In-Competition* or *Out-of-Competition*), or other doping violation occurred, through the commencement of any *Provisional Suspension* or *Ineligibility* period, shall, unless fairness requires otherwise, be *Disqualified* with all of the resulting consequences including forfeiture of any medals, points and prizes.

10.8 Commencement of Ineligibility Period

The period of *Ineligibility* shall start on the date of the hearing decision providing for *Ineligibility* or, if the hearing is waived, on the date *Ineligibility* is accepted or otherwise imposed. Any period of *Provisional Suspension* (whether imposed or voluntarily accepted) shall be credited against

10.8 Comment: Currently, many Anti-Doping Organizations start the two-year period of *Ineligibility* at the time a hearing decision is rendered. Those Anti-Doping Organizations also

frequently invalidate results retroactively to the date a positive *Sample* was collected. Other Anti-Doping Organizations simply start the two-year suspension on the date the

the total period of *Ineligibility* to be served. Where required by fairness, such as delays in the hearing process or other aspects of *Doping Control* not attributable to the *Athlete*, the body imposing the sanction may start the period of *Ineligibility* at an earlier date commencing as early as the date of *Sample* collection.

10.9 Status During Ineligibility

No *Person* who has been declared *Ineligible* may, during the period of *Ineligibility*, participate in any capacity in a *Competition* or activity (other than authorized anti-doping education or rehabilitation programs) authorized or organized by any *Signatory* or *Signatory's* member organization. In addition, for any anti-doping rule violation not involving specified substances described in Article 10.3, some or all sport-related financial support or other sport-related benefits received by such *Person* will be withheld by *Signatories*. *Signatories'* member organizations and governments. A *Person* subject to a period of *Ineligibility* longer than four years may, after completing four years of the period of *Ineligibility*,

positive *Sample* was collected. The OMADC, as clarified by its Explanatory Document, does not mandate either approach. The approach provided in the Code gives *Athletes* a strong disincentive to drag out the hearing process while they compete in the interim. It also encourages them to voluntarily accept *Provisional Suspensions* pending a hearing. On the other hand, the body imposing the sanction can start the sanction running before the date the hearing decision is reached so that an *Athlete* is not penalized by delays in the *Doping Control* process which are not his or her fault, for example, inordinate delay by the laboratory in reporting a positive test or delays in scheduling the hearing caused by the Anti-Doping Organization.

10.9 Comment: The rules of some Anti-Doping Organizations only ban an *Athlete* from "competing" during a period of *Ineligibility*. For example, an *Athlete* in those sports could still coach during the *Ineligibility* period. This Article adopts the position set forth in the OMADC that an *Athlete* who is made ineligible for doping should not participate in any capacity in an authorized Event or activity during the *Ineligibility* period. This would preclude, for example, practicing with a national team, or acting as a coach or sport official. Sanctions in one sport will also be recognized by other sports (see Article 15.4). This article would not prohibit the *Person* from participating in sport on a purely recreational level.

participate in local sport events in a sport other than the sport in which the *Person* committed the anti-doping rule violation, but only so long as the local sport event is not at a level that could otherwise qualify such *Person* directly or indirectly to compete in (or accumulate points toward) a national championship or *International Event*.

10.10 Reinstatement Testing

As a condition to regaining eligibility at the end of a specified period of *Ineligibility*, an *Athlete* must, during any period of *Provisional Suspension* or *Ineligibility*, make him or herself available for *Out-of-Competition Testing* by any *Anti-Doping Organization* having testing jurisdiction, and must, if requested, provide current and accurate whereabouts information. If an *Athlete* subject to a period of *Ineligibility* retires from sport and is removed from *Out-of-Competition Testing* pools and later seeks reinstatement, the *Athlete* shall not be eligible for reinstatement until the *Athlete* has notified relevant *Anti-Doping Organizations* and has been subject to *Out-of-Competition Testing* for a period of time equal to the period of *Ineligibility* remaining as of the date the *Athlete* had retired.

ARTICLE 11 CONSEQUENCES TO TEAMS

Where more than one team member in a *Team Sport* has been notified of a possible anti-doping rule violation under Article 7 in connection with an *Event*, the Team shall be subject to *Target Testing* for the *Event*. If more than one team member in a *Team Sport* is found to have committed an anti-doping rule violation during the *Event*, the team may be subject to *Disqualification* or other disciplinary action. In sports which are not *Team Sports* but

10.10 Comment: On a related issue, the Code does not establish a rule, but rather leaves it to the various Anti-Doping Organizations to establish their own rules addressing eligibility

requirements for Athletes who are not ineligible and retire from sport while included in an Out-of-Competition pool and then seek to return to active participation in sport.

where awards are given to teams, *Disqualification* or other disciplinary action against the team when one or more team members have committed an anti-doping rule violation shall be as provided in the applicable rules of the International Federation.

ARTICLE 12 SANCTIONS AGAINST SPORTING BODIES

Nothing in this *Code* precludes any *Signatory* or government accepting the *Code* from enforcing its own rules for the purpose of imposing sanctions on another sporting body over which the *Signatory* or government has authority.

ARTICLE 13 APPEALS

13.1 Decisions Subject to Appeal

Decisions made under the *Code* or rules adopted pursuant to the *Code* may be appealed as set forth below in Articles 13.2 through 13.4. Such decisions shall remain in effect while under appeal unless the appellate body orders otherwise. Before an appeal is commenced, any post-decision review provided in the *Anti-Doping Organization's* rules must be exhausted, provided that such review respects the principles set forth in Article 13.2.2 below.

13.2 Appeals from Decisions Regarding Anti-Doping Rule Violations, Consequences, and Provisional Suspensions

A decision that an anti-doping rule violation was committed, a decision imposing *Consequences* for an anti-doping rule violation, a decision that no anti-doping rule violation was committed, a decision that an *Anti-Doping Organization* lacks jurisdiction to rule on an alleged anti-doping rule violation or its *Consequences*.

12 Comment: This Article makes it clear that the Code does not restrict whatever disciplinary rights between organizations may otherwise exist.

13.1 Comment: The comparable OMADC Article is broader in that it provides that any dispute arising out of the application of the OMADC may be appealed to CAS.

and a decision to impose a *Provisional Suspension* as a result of a *Provisional Hearing* or in violation of Article 7.5 may be appealed exclusively as provided in this Article 13.2.

13.2.1 Appeals Involving *International-Level Athletes*

In cases arising from competition in an *International Event* or in cases involving *International-Level Athletes*, the decision may be appealed exclusively to the Court of Arbitration for Sport ("CAS") in accordance with the provisions applicable before such court.

13.2.2 Appeals Involving *National-Level Athletes*

In cases involving national-level *Athletes*, as defined by each *National Anti-Doping Organization*, that do not have a right to appeal under Article 13.2.1, the decision may be appealed to an independent and impartial body in accordance with rules established by the *National Anti-Doping Organization*. The rules for such appeal shall respect the following principles:

- A timely hearing;
- Fair, impartial and independent hearing body;
- The right to be represented by counsel at the *Person's* own expense; and
- A timely, written, reasoned decision.

13.2.3 *Persons* Entitled to Appeal

In cases under Article 13.2.1, the following parties shall have the right to appeal to CAS: (a) the *Athlete*

13.2.1 Comment: CAS decisions are final and binding except for any review required by law applicable to the annulment or enforcement of arbitral awards.

13.2.2 Comment: An *Anti-Doping Organization* may elect to comply with this Article by giving its national-level *Athletes* the right to appeal directly to CAS.

or other *Person* who is the subject of the decision being appealed; (b) the other party to the case in which the decision was rendered; (c) the relevant International Federation and any other *Anti-Doping Organization* under whose rules a sanction could have been imposed; (d) the International Olympic Committee or International Paralympic Committee, as applicable, where the decision may have an effect in relation to the Olympic Games or Paralympic Games, including decisions affecting eligibility for the Olympic Games or Paralympic Games; and (e) WADA. In cases under Article 13.2.2, the parties having the right to appeal to the national-level reviewing body shall be as provided in the *National Anti-Doping Organization's* rules but, at a minimum, shall include: (a) the *Athlete* or other *Person* who is the subject of the decision being appealed; (b) the other party to the case in which the decision was rendered; (c) the relevant International Federation; and (d) WADA. For cases under Article 13.2.2, WADA and the International Federation shall also have the right to appeal to CAS with respect to the decision of the national-level reviewing body.

Notwithstanding any other provision herein, the only *Person* that may appeal from a *Provisional Suspension* is the *Athlete* or other *Person* upon whom the *Provisional Suspension* is imposed.

13.3 Appeals from Decisions Granting or Denying a Therapeutic Use Exemption

Decisions by WADA reversing the grant or denial of a therapeutic use exemption may be appealed exclusively to CAS by the *Athlete* or the *Anti-Doping Organization* whose decision was reversed. Decisions by *Anti-Doping Organizations* other than WADA denying therapeutic use exemptions, which are not reversed by WADA, may be appealed by *International-Level Athletes* to CAS and by

other *Athletes* to the national level reviewing body described in Article 13.2.2. If the national level reviewing body reverses the decision to deny a therapeutic use exemption, that decision may be appealed to CAS by WADA.

13.4 Appeals from Decisions Imposing *Consequences* under Part Three of the *Code*

With respect to *consequences* imposed under Part Three (Roles and Responsibilities) of the *Code*, the entity upon which *consequences* are imposed under Part Three of the *Code* shall have the right to appeal exclusively to CAS in accordance with the provisions applicable before such court.

13.5 Appeals from Decisions Suspending or Revoking Laboratory Accreditation

Decisions by WADA to suspend or revoke a laboratory's WADA accreditation may be appealed only by that laboratory with the appeal being exclusively to CAS.

ARTICLE 14 CONFIDENTIALITY AND REPORTING

The *Signatories* agree to the principles of coordination of anti-doping results, public transparency and accountability and respect for the privacy interests of individuals alleged to have violated anti-doping rules as provided below:

14.1 Information Concerning *Adverse Analytical Findings* and Other Potential Anti-Doping Rule Violations

An *Athlete* whose *Sample* has resulted in an *Adverse Analytical Finding*, or an *Athlete* or other *Person* who may

13.5 Comment: The object of the *Code* is to have anti-doping matters resolved through fair and transparent internal processes with a final appeal. Anti-doping decisions by Anti-Doping Organizations are made transparent in Article 14. Specified Persons and organizations, including WADA, are

then given the opportunity to appeal those decisions. Note, that the definition of interested Persons and organizations with a right to appeal under Article 13 does not include Athletes, or their federations, who might benefit from having another competitor disqualified.

have violated an anti-doping rule, shall be notified by the *Anti-Doping Organization* with results management responsibility as provided in Article 7 (Results Management). The *Athlete's National Anti-Doping Organization* and International Federation and WADA shall also be notified not later than the completion of the process described in Articles 7.1 and 7.2. Notification shall include: the *Athlete's* name, country, sport and discipline within the sport, whether the test was *In-Competition* or *Out-of-Competition*, the date of *Sample* collection and the analytical result reported by the laboratory. The same *Persons* and *Anti-Doping Organizations* shall be regularly updated on the status and findings of any review or proceedings conducted pursuant to Articles 7 (Results Management), 8 (Right to a Fair Hearing) or 13 (Appeals), and, in any case in which the period of *Ineligibility* is eliminated under Article 10.5.1 (*No Fault or Negligence*), or reduced under Article 10.5.2 (*No Significant Fault or Negligence*), shall be provided with a written reasoned decision explaining the basis for the elimination or reduction. The recipient organizations shall not disclose this information beyond those persons within the organization with a need to know until the *Anti-Doping Organization* with results management responsibility has made public disclosure or has failed to make public disclosure as required in Article 14.2 below.

14.2 Public Disclosure

The identity of *Athletes* whose *Samples* have resulted in *Adverse Analytical Findings*, or *Athletes* or other *Persons* who were alleged by an *Anti-Doping Organization* to have violated other anti-doping rules, may be publicly disclosed by the *Anti-doping Organization* with results management responsibility no earlier than completion of the administrative review described in Articles 7.1 and 7.2. No later than twenty days after it has been determined in a hearing in accordance with Article 8 that an anti-doping rule violation has occurred, or such hearing has been

waived, or the assertion of an anti-doping rule violation has not been timely challenged, the *Anti-Doping Organization* responsible for results management must publicly report the disposition of the anti-doping matter.

14.3 Athlete Whereabouts Information

Athletes who have been identified by their International Federation or *National Anti-Doping Organization* for inclusion in an *Out-of-Competition Testing* pool shall provide accurate, current location information. The International Federations and *National Anti-Doping Organizations* shall coordinate the identification of *Athletes* and the collecting of current location information and shall submit it to WADA. WADA shall make this information accessible to other *Anti-Doping Organizations* having authority to test the *Athlete* as provided in Article 15. This information shall be maintained in strict confidence at all times; shall be used exclusively for purposes of planning, coordinating or conducting *Testing*; and shall be destroyed after it is no longer relevant for these purposes.

14.4 Statistical Reporting

Anti-Doping Organizations shall, at least annually, publish publicly a general statistical report of their *Doping Control* activities with a copy provided to WADA.

14.5 Doping Control Information Clearing House

WADA shall act as a central clearing house for *Doping Control Testing* data and results for *International-Level Athletes* and national-level *Athletes* that have been included in their *National Anti-Doping Organization's Registered Testing Pool*. To facilitate coordinated test distribution planning and to avoid unnecessary duplication in *Testing* by the various *Anti-Doping Organizations*, each *Anti-Doping Organization* shall report all *In-Competition* and *Out-of-Competition* tests on such *Athletes* to the WADA clearinghouse as soon as possible after such tests have been conducted. WADA shall make this information accessible to the *Athlete*, the *Athlete's National Federation*, *National Olympic Committee* or *National*

Paralympic Committee, *National Anti-Doping Organization*, *International Federation*, and the *International Olympic Committee* or *International Paralympic Committee*. Private information regarding an *Athlete* shall be maintained by WADA in strict confidence. WADA shall, at least annually, publish statistical reports summarizing such information.

ARTICLE 15: CLARIFICATION OF DOPING CONTROL RESPONSIBILITIES

15.1 Event Testing

The collection of *Samples for Doping Control* does and should take place at both *International Events* and *National Events*. However, only a single organization should be responsible for initiating and directing *Testing* during an *Event*. At *International Events*, the collection of *Doping Control Samples* shall be initiated and directed by the international organization which is the ruling body for the *Event* (e.g., the IOC for the Olympic Games, the International Federation for a World Championship, and PASO for the Pan American Games). If the international organization decides not to conduct any *Testing* at such an *Event*, the *National Anti-Doping Organization* for the country where the *Event* occurs may, in coordination with and with the approval of the international organization or WADA, initiate and conduct such *Testing*. At *National Events*, the collection of *Doping Control Samples* shall be initiated and directed by the designated *National Anti-Doping Organization* of that country.

15 Comment: To be effective, the anti-doping effort must involve many *Anti-Doping Organizations* conducting strong programs at both the international and national levels. Rather than limiting the responsibilities of one group in favor of the exclusive competency of the other, the Code manages potential problems associated with overlapping responsibilities, first by creating a much higher level of overall harmonization

and second, by establishing rules of precedence and cooperation in specific areas.

15.1 Comment: The *Anti-Doping Organization* "initiating and directing testing" may, if it chooses, enter into agreements with other organizations to which it delegates responsibility for Sample collection or other aspects of the *Doping Control* process.

15.2 Out-of-Competition Testing

Out-of-Competition Testing is and should be initiated and directed by both international and national organizations. *Out-of-Competition Testing* may be initiated and directed by: (a) WADA; (b) the IOC or IPC in connection with the Olympic Games or Paralympic Games; (c) the *Athlete's* International Federation; (d) the *Athlete's National Anti-Doping Organization*; or (e) the *National Anti-Doping Organization* of any country where the *Athlete* is present. *Out-of-Competition Testing* should be coordinated through WADA in order to maximize the effectiveness of the combined *Testing* effort and to avoid unnecessary repetitive *Testing* of individual *Athletes*.

15.3 Results Management, Hearings and Sanctions

Except as provided in Article 15.3.1 below, results management and hearings shall be the responsibility of and shall be governed by the procedural rules of the *Anti-Doping Organization* that initiated and directed *Sample* collection (or, if no *Sample* collection is involved, the organization which discovered the violation). Regardless of which organization conducts results management or hearings, the principles set forth in Articles 7 and 8 shall be respected and the rules identified in the Introduction to Part One to be incorporated without substantive change must be followed.

15.3.1 Results management and the conduct of hearings for an anti-doping rule violation arising from a test by, or discovered by, a *National Anti-Doping Organization* involving an *Athlete* that is not a citizen

15.2 Comment: Additional authority to conduct *Testing* may be authorized by means of bilateral or multilateral agreements among Signatories and governments.

15.3 Comment: In some cases, the procedural rules of the *Anti-Doping Organization* which initiated and directed the *Sample* collection may

specify that results management will be handled by another organization (e.g., the *Athlete's* national federation). In such event, it shall be the *Anti-Doping Organization's* responsibility to confirm that the other organization's rules are consistent with the Code.

15.3.1 Comment: No absolute rule is established for managing results and

or resident of that country shall be administered as directed by the rules of the applicable International Federation. Results management and the conduct of hearings from a test by the International Olympic Committee, the International Paralympic Committee, or a *Major Event Organization*, shall be referred to the applicable International Federation as far as sanctions beyond *Disqualification* from the *Event* or the results of the *Event*.

15.4 Mutual Recognition

Subject to the right to appeal provided in Article 13, the *Testing*, therapeutic use exemptions and hearing results or other final adjudications of any *Signatory* which are consistent with the *Code* and are within that *Signatory's* authority, shall be recognized and respected by all other *Signatories*. *Signatories* may recognize the same actions of other bodies which have not accepted the *Code* if the rules of those bodies are otherwise consistent with the *Code*.

ARTICLE 16: DOPING CONTROL FOR ANIMALS COMPETING IN SPORT

16.1 In any sport that includes animals in competition, the International Federation for that sport shall establish and implement anti-doping rules for the animals included in that sport. The anti-doping rules shall include a list of *Prohibited Substances*, appropriate *Testing* procedures and a list of approved laboratories for *Sample* analysis.

conducting hearings where a *National Anti-Doping Organization* tests a foreign national athlete over whom it would have had no jurisdiction but for the *Athlete's* presence in the *National Anti-Doping Organization's* country. Under this Article, it is left to the International Federation to determine under its own rules whether, for example, management of the case

should be referred to the *Athlete's* *National Anti-Doping Organization*, remain with the *Anti-Doping Organization* that collected the *Sample*, or be taken over by the International Federation.

- 16.2** With respect to determining anti-doping rule violations, results management, fair hearings, *Consequences*, and appeals for animals involved in sport, the International Federation for that sport shall establish and implement rules that are generally consistent with Articles 1, 2, 3, 9, 10, 11, 13 and 17 of the *Code*.

ARTICLE 17: STATUTE OF LIMITATIONS

No action may be commenced against an *Athlete* or other *Person* for a violation of an anti-doping rule contained in the *Code* unless such action is commenced within eight years from the date the violation occurred.

17 Comment: *This does not restrict the Anti-Doping Organization from considering an earlier anti-doping violation for purposes of the sanction for a subsequent violation that occurs more than eight years later. In other words, a second violation ten years after a first violation is considered a second violation for sanction purposes.*

PART TWO

EDUCATION & RESEARCH

ARTICLE 18: EDUCATION**18.1 Basic Principle and Primary Goal**

The basic principle for information and education programs shall be to preserve the spirit of sport as described in the Introduction to the *Code*, from being undermined by doping. The primary goal shall be to dissuade *Athletes* from using *Prohibited Substances* and *Prohibited Methods*.

18.2 Program and Activities

Each *Anti-Doping Organization* should plan, implement and monitor information and education programs. The programs should provide *Participants* with updated and accurate information on at least the following issues:

- Substances and methods on the *Prohibited List*
- Health consequences of doping
- *Doping Control* procedures
- *Athletes'* rights and responsibilities

The programs should promote the spirit of sport in order to establish an anti-doping environment which influences behavior among *Participants*.

Athlete Support Personnel should educate and counsel *Athletes* regarding anti-doping policies and rules adopted pursuant to the *Code*.

18.3 Coordination and Cooperation

All *Signatories* and *Participants* shall cooperate with each other and governments to coordinate their efforts in anti-doping information and education.

ARTICLE 19: RESEARCH**19.1 Purpose of Anti-Doping Research**

Anti-doping research contributes to the development and implementation of efficient programs within *Doping Control* and to anti-doping information and education.

19.2 Types of Research

Anti-doping research may include, for example, sociological, behavioral, juridical and ethical studies in addition to medical, analytical and physiological investigation.

19.3 Coordination

Coordination of anti-doping research through WADA is encouraged. Subject to intellectual property rights, copies of anti-doping research results should be provided to WADA.

19.4 Research Practices

Anti-doping research shall comply with internationally recognized ethical practices.

19.5 Research Using *Prohibited Substances* and *Prohibited Methods*

Research efforts should avoid the administration of *Prohibited Substances* or *Prohibited Methods* to *Athletes*.

19.6 Misuse of Results

Adequate precautions should be taken so that the results of anti-doping research are not misused and applied for doping.

PART THREE

ROLES & RESPONSIBILITIES

ARTICLE 20: ADDITIONAL ROLES AND RESPONSIBILITIES OF SIGNATORIES

20.1 Roles and Responsibilities of the International Olympic Committee

20.1.1 To adopt and implement anti-doping policies and rules for the Olympic Games which conform with the *Code*.

20.1.2 To require as a condition of recognition by the International Olympic Committee, that International Federations within the Olympic Movement are in compliance with the *Code*.

20.1.3 To withhold some or all Olympic funding of sport organizations that are not in compliance with the *Code*.

20.1.4 To take appropriate action to discourage non-compliance with the *Code* as provided in Article 23.5.

20.1.5 To authorize and facilitate the *Independent Observer Program*.

20.2 Roles and Responsibilities of the International Paralympic Committee

20.2.1 To adopt and implement anti-doping policies and rules for the Paralympic Games which conform with the *Code*.

20.2.2 To require as a condition of recognition by the International Paralympic Committee, that National Paralympic Committees within the Olympic Movement are in compliance with the *Code*.

20 Comment: Responsibilities for Signatories and Participants are addressed in various articles in the

Code and the responsibilities listed in this part are additional to these responsibilities.

20.2.3 To withhold some or all Paralympic funding of sport organizations that are not in compliance with the *Code*.

20.2.4 To take appropriate action to discourage non-compliance with the *Code* as provided in Article 23.5.

20.2.5 To authorize and facilitate the *Independent Observer Program*.

20.3 Roles and Responsibilities of International Federations

20.3.1 To adopt and implement anti-doping policies and rules which conform with the *Code*.

20.3.2 To require as a condition of membership that the policies, rules and programs of National Federations are in compliance with the *Code*.

20.3.3 To require all *Athletes* and *Athlete Support Personnel* within their jurisdiction to recognize and be bound by anti-doping rules in conformance with the *Code*.

20.3.4 To require *Athletes* who are not regularly members of the International Federation or one of its member National Federations to be available for *Sample* collection and provide accurate and up-to-date whereabouts information if required by the conditions for eligibility established by the International Federation or, as applicable, the *Major Event Organization*.

20.3.5 To monitor the anti-doping programs of National Federations.

20.3.4 Comment: This would include, for example, *Athletes* from professional leagues.

- 20.3.6** To take appropriate action to discourage non-compliance with the *Code* as provided in Article 23.5.
- 20.3.7** To authorize and facilitate the *Independent Observer* program at *International Events*.
- 20.3.8** To withhold some or all funding to its member National Federations that are not in compliance with the *Code*.
- 20.4** Roles and Responsibilities of *National Olympic Committees* and *National Paralympic Committees*
- 20.4.1** To ensure that their anti-doping policies and rules conform with the *Code*.
- 20.4.2** To require as a condition of membership or recognition that National Federations' anti-doping policies and rules are in compliance with the applicable provisions of the *Code*.
- 20.4.3** To require *Athletes* who are not regular members of a National Federation to be available for *Sample* collection and provide accurate and up-to-date whereabouts information on a regular basis if required during the year before the Olympic Games as a condition of participation in the Olympic Games.
- 20.4.4** To cooperate with their *National Anti-Doping Organization*.
- 20.4.5** To withhold some or all funding, during any period of his or her *Ineligibility*, to any *Athlete* or *Athlete Support Personnel* who has violated anti-doping rules.
- 20.4.6** To withhold some or all funding to its member or recognized National Federations that are not in compliance with the *Code*.

- 20.5** Roles and Responsibilities of *National Anti-Doping Organizations*
- 20.5.1** To adopt and implement anti-doping rules and policies which conform with the *Code*.
- 20.5.2** To cooperate with other relevant national organizations and other *Anti-Doping Organizations*.
- 20.5.3** To encourage reciprocal testing between *National Anti-Doping Organizations*.
- 20.5.4** To promote anti-doping research.
- 20.6** Roles and Responsibilities of *Major Event Organizations*
- 20.6.1** To adopt and implement anti-doping policies and rules for their *Events* which conform with the *Code*.
- 20.6.2** To take appropriate action to discourage non-compliance with the *Code* as provided in Article 23.5.
- 20.6.3** To authorize and facilitate the *Independent Observer Program*.
- 20.7** Roles and Responsibilities of *WADA*
- 20.7.1** To adopt and implement policies and procedures which conform with the *Code*.
- 20.7.2** To monitor the processing of *Adverse Analytical Findings*.
- 20.7.3** To approve *International Standards* applicable to the implementation of the *Code*.
- 20.7.4** To accredit laboratories to conduct *Sample* analysis or to approve others to conduct *Sample* analysis.

- 20.7.5 To develop and approve Models of Best Practice.
- 20.7.6 To promote, conduct, commission, fund and coordinate anti-doping research.
- 20.7.7 To conduct an effective *Independent Observer Program*.
- 20.7.8 To conduct *Doping Controls* as authorized by other *Anti-Doping Organizations*.

ARTICLE 21: ROLES AND RESPONSIBILITIES OF PARTICIPANTS

21.1 Roles and Responsibilities of *Athletes*

- 21.1.1 To be knowledgeable of and comply with all applicable anti-doping policies and rules adopted pursuant to the *Code*.
- 21.1.2 To be available for *Sample* collection.
- 21.1.3 To take responsibility, in the context of anti-doping, for what they ingest and use.
- 21.1.4 To inform medical personnel of their obligation not to *Use Prohibited Substances* and *Prohibited Methods* and to take responsibility to make sure that any medical treatment received does not violate anti-doping policies and rules adopted pursuant to the *Code*.

21.2 Roles and Responsibilities of *Athlete Support Personnel*

- 21.2.1 To be knowledgeable of and comply with all anti-doping policies and rules adopted pursuant to the *Code* and which are applicable to them or the *Athletes* whom they support.
- 21.2.2 To cooperate with the *Athlete Testing* program.

- 21.2.3 To use their influence on *Athlete* values and behavior to foster anti-doping attitudes.

ARTICLE 22: INVOLVEMENT OF GOVERNMENTS

Each government's commitment to the *Code* will be evidenced by its signing a Declaration on or before the first day of the Athens Olympic Games to be followed by a process leading to a convention or other obligation to be implemented as appropriate to the constitutional and administrative contexts of each government on or before the first day of the Turin Winter Olympic Games.

It is the expectation of the *Signatories* that the Declaration and the convention or other obligation will reflect the following major points:

- 22.1 Affirmative measures will be undertaken by each government in support of anti-doping in at least the following areas:
 - Support for national anti-doping programs;
 - The availability of *Prohibited Substances* and *Prohibited Methods*;
 - Facilitate access for WADA to conduct *Out-of-Competition Doping Controls*;
 - The problem of nutritional supplements which contain undisclosed *Prohibited Substances*; and
 - Withholding some or all financial support from sport organizations and *Participants* that are not in compliance with the *Code* or applicable anti-doping rules adopted pursuant to the *Code*.

22 Comment: Most governments cannot be parties to, or be bound by, private non-governmental instruments such as the *Code*. For that reason, governments are not asked to be *Signatories* to the *Code*. However, the effort to combat doping through the coordinated and harmonized program reflected in the *Code* is very much a

joint effort between the sport movement and governments. An example of one type of obligation referred to above is the convention discussed in the Final Communiqué of the UNESCO Round Table of Ministers and Senior Officials Responsible for Physical Education and Sport held in Paris on 9/10 January 2003.

- 22.2** All other governmental involvement with anti-doping will be brought into harmony with the *Code*.
- 22.3** Ongoing compliance with the commitments reflected in the convention or other obligation will be monitored as determined in consultation between *WADA* and the applicable government(s).

PART FOUR

ACCEPTANCE, COMPLIANCE, MODIFICATION & INTERPRETATION

ARTICLE 23: ACCEPTANCE, COMPLIANCE AND MODIFICATION**23.1 Acceptance of the Code**

23.1.1 The following entities shall be *Signatories* accepting the Code: WADA, The International Olympic Committee, International Federations, The International Paralympic Committee, *National Olympic Committees*, National Paralympic Committees, *Major Event Organizations*, and *National Anti-Doping Organizations*. These entities shall accept the Code by signing a declaration of acceptance upon approval by each of their respective governing bodies.

23.1.2 Other sport organizations that may not be under the control of a *Signatory* may, upon WADA's invitation, also accept the Code.

23.1.3 A list of all acceptances will be made public by WADA.

23.2 Implementation of the Code

23.2.1 The *Signatories* shall implement applicable Code provisions through policies, statutes, rules or regulations according to their authority and within their relevant spheres of responsibility.

23.1.1 Comment: Each accepting Signatory will separately sign an identical copy of the standard form common declaration of acceptance and deliver it to WADA. The act of acceptance will be as authorized by the organic documents of each organization. For example, an International Federation by its Congress and WADA by its Foundation Board.

23.1.2 Comment: Those professional leagues that are not currently under the jurisdiction of any government or International Federation will be encouraged to accept the Code.

23.2.2 In implementing the Code, the *Signatories* are encouraged to use the Models of Best Practice recommended by WADA.

23.3 Acceptance and Implementation Deadlines

23.3.1 *Signatories* shall accept and implement the Code on or before the first day of the Athens Olympic Games.

23.3.2 The Code may be accepted after the above-referenced deadlines; however, *Signatories* shall not be considered in compliance with the Code until they have accepted the Code (and that acceptance has not been withdrawn).

23.4 Monitoring Compliance with the Code

23.4.1 Compliance with the Code shall be monitored by WADA or as otherwise agreed by WADA.

23.4.2 To facilitate monitoring, each *Signatory* shall report to WADA on its compliance with the Code every second year and shall explain reasons for noncompliance.

23.4.3 WADA shall consider explanations for non-compliance and, in extraordinary situations, may recommend to the International Olympic Committee, International Paralympic Committee, International Federations, and *Major Event Organizations* that they provisionally excuse the non-compliance.

23.4.3 Comment: WADA recognizes that amongst Signatories and governments, there will be significant differences in anti-doping experience, resources, and the legal context in

which anti-doping activities are carried out. In considering whether an organization is compliant, WADA will consider these differences.

23.4.4 WADA shall, after dialogue with the subject organization, make reports on compliance to the International Olympic Committee, the International Paralympic Committee, International Federations, and *Major Event Organizations*. These reports shall also be made available to the public.

23.5 Consequences of Noncompliance with the *Code*

23.5.1 Noncompliance with the *Code* by either the government or *National Olympic Committee* of a country may result in consequences with respect to Olympic Games, Paralympic Games, World Championships or the *Events of Major Event Organizations* as determined by the ruling body for each *Event*. The imposition of such consequences may be appealed by the *National Olympic Committee* or government to CAS pursuant to Article 13.4.

23.6 Modification of the *Code*

23.6.1 WADA shall be responsible for overseeing the evolution and improvement of the *Code*. *Athletes* and all *Signatories* and governments shall be invited to participate in such process.

23.6.2 WADA shall initiate proposed amendments to the *Code* and shall ensure a consultative process to both receive and respond to recommendations and to facilitate review and feedback from *Athletes*, *Signatories* and governments on recommended amendments.

23.6.3 Amendments to the *Code* shall, after appropriate consultation, be approved by a two-thirds majority of the WADA Foundation Board including a majority of both the public sector and Olympic Movement members casting votes. Amendments shall, unless provided otherwise, go into effect three months after such approval.

23.6.4 *Signatories* shall implement any applicable amendment to the *Code* within one year of approval by the WADA Foundation Board.

23.7 Withdrawal of Acceptance of the *Code*

23.7.1 *Signatories* may withdraw acceptance of the *Code* after providing WADA six-month's written notice of their intent to withdraw.

ARTICLE 24: INTERPRETATION OF THE CODE

24.1 The official text of the *Code* shall be maintained by WADA and shall be published in English and French. In the event of any conflict between the English and French versions, the English version shall prevail.

24.2 The comments annotating various provisions of the *Code* are included to assist in the understanding and interpretation of the *Code*.

24.3 The *Code* shall be interpreted as an independent and autonomous text and not by reference to the existing law or statutes of the *Signatories* or governments.

- 24.4** The headings used for the various Parts and Articles of the *Code* are for convenience only and shall not be deemed part of the substance of the *Code* or to affect in any way the language of the provisions to which they refer.
- 24.5** The *Code* shall not apply retrospectively to matters pending before the date the *Code* is accepted by a Signatory and implemented in its rules.
- 24.6** APPENDIX I Definitions shall be considered an integral part of the *Code*.

24.5 Comment: For example, conduct which is an anti-doping rule violation described in the Code, but which is not a violation under an International Federation's pre-Code rules, would not be a violation until the International Federation's rules are changed.

Pre-Code anti-doping rule violations would continue to count as "First violations" or "Second violations" for purposes of determining sanctions under Article 10 for subsequent post-Code violations.

APPENDIX 1

DEFINITIONS

Adverse Analytical Finding: A report from a laboratory or other approved *Testing* entity that identifies in a *Specimen* the presence of a *Prohibited Substance* or its *Metabolites* or *Markers* (including elevated quantities of endogenous substances) or evidence of the *Use of a Prohibited Method*.

Anti-Doping Organization: A *Signatory* that is responsible for adopting rules for initiating, implementing or enforcing any part of the *Doping Control* process. This includes, for example, the International Olympic Committee, the International Paralympic Committee, other *Major Event Organizations* that conduct *Testing* at their *Events*, WADA, International Federations, and *National Anti-Doping Organizations*.

Athlete: For purposes of *Doping Control*, any *Person* who participates in sport at the international level (as defined by each International Federation) or national level (as defined by each *National Anti-Doping Organization*) and any additional *Person* who participates in sport at a lower level if designated by the *Person's National Anti-Doping Organization*. For purposes of anti-doping information and education, any *Person* who participates in sport under the authority of any *Signatory*, government, or other sports organization accepting the *Code*.

Athlete Support Personnel: Any coach, trainer, manager, agent, team staff, official, medical or para-medical personnel working with or treating *Athletes* participating in or preparing for sports competition.

Athlete Comment: This definition makes it clear that all international and national-calibre athletes are subject to the anti-doping rules of the *Code*, with the precise definitions of international and national level sport to be set forth in the anti-doping rules of the International Federations and *National Anti-Doping Organizations*, respectively. At the national level, anti-doping rules adopted pursuant to the *Code* shall apply, at a minimum, to all persons on national teams and all

persons qualified to compete in any national championship in any sport. The definition also allows each *National Anti-Doping Organization*, if it chooses to do so, to expand its anti-doping control program beyond national-calibre athletes to athletes at lower levels of competition. Athletes at all levels of competition should receive the benefit of anti-doping information and education.

Attempt: Purposely engaging in conduct that constitutes a substantial step in a course of conduct planned to culminate in the commission of an anti-doping rule violation. Provided, however, there shall be no anti-doping rule violation based solely on an *Attempt* to commit a violation if the *Person* renounces the attempt prior to it being discovered by a third party not involved in the *Attempt*.

Code: The World Anti-Doping Code.

Competition: A single race, match, game or singular athletic contest. For example, the finals of the Olympic 100-meter dash. For stage races and other athletic contests where prizes are awarded on a daily or other interim basis the distinction between a *Competition* and an *Event* will be as provided in the rules of the applicable International Federation.

Consequences of Anti-Doping Rules Violations: An *Athlete's* or other *Person's* violation of an anti-doping rule may result in one or more of the following: (a) **Disqualification** means the *Athlete's* results in a particular *Competition* or *Event* are invalidated, with all resulting consequences including forfeiture of any medals, points and prizes; (b) **Ineligibility** means the *Athlete* or other *Person* is barred for a specified period of time from participating in any *Competition* or other activity or funding as provided in Article 10.9; and (c) **Provisional Suspension** means the *Athlete* or other *Person* is barred temporarily from participating in any *Competition* prior to the final decision at a hearing conducted under Article 8 (Right to a Fair Hearing).

Disqualification: See *Consequences of Anti-Doping Rules Violations* above.

Doping Control: The process including test distribution planning, *Sample* collection and handling, laboratory analysis, results management, hearings and appeals.

Event: A series of individual *Competitions* conducted together under one ruling body (e.g., the Olympic Games, FINA World Championships, or Pan American Games).

In-Competition: For purposes of differentiating between *In-Competition* and *Out-of-Competition Testing*, unless provided otherwise in the rules of an International Federation or other relevant *Anti-Doping Organization*, an *In-Competition* test is a test where an *Athlete* is selected for testing in connection with a specific *Competition*.

Independent Observer Program: A team of observers, under the supervision of WADA, who observe the *Doping Control* process at certain *Events* and report on observations. If WADA is testing *In-Competition* at an *Event*, the observers shall be supervised by an independent organization.

Ineligibility: See *Consequences of Anti-Doping Rules Violations* above.

International Event: An *Event* where the International Olympic Committee, the International Paralympic Committee, an International Federation, a *Major Event Organization*, or another international sport organization is the ruling body for the *Event* or appoints the technical officials for the *Event*.

International-Level Athlete: *Athletes* designated by one or more International Federations as being within the *Registered Testing Pool* for an International Federation.

International Standard: A standard adopted by WADA in support of the *Code*. Compliance with an *International Standard* (as opposed to another alternative standard, practice or procedure) shall be sufficient to conclude that the procedures addressed by the *International Standard* were performed properly.

In-Competition Comment: The distinction between "In-Competition" and "Out-of-Competition" testing is significant because the full *Prohibited List* is only tested for "In-Competition." *Prohibited stimulants*, for example, are not tested for *Out-of-Competition* because they have no performance enhancing benefit unless they are in

the *Athlete's* system while the *Athlete* is actually competing. So long as the *prohibited stimulant* has cleared the *Athlete's* system at the time the *Athlete* competes, it makes no difference whether that stimulant could have been found in the *Athlete's* urine the day before or the day after the *Competition*.

Major Event Organizations: This term refers to the continental associations of *National Olympic Committees* and other international multi-sport organizations that function as the ruling body for any continental, regional or other *International Event*.

Marker: A compound, group of compounds or biological parameters that indicates the *Use* of a *Prohibited Substance* or *Prohibited Method*.

Metabolite: Any substance produced by a biotransformation process.

Minor: A natural *Person* who has not reached the age of majority as established by the applicable laws of his or her country of residence.

National Anti-Doping Organization: The entity(ies) designated by each country as possessing the primary authority and responsibility to adopt and implement anti-doping rules, direct the collection of *Samples*, the management of test results, and the conduct of hearings, all at the national level. If this designation has not been made by the competent public authority(ies), the entity shall be the country's *National Olympic Committee* or its designee.

National Event: A sport *Event* involving international or national-level *Athletes* that is not an *International Event*.

National Olympic Committee: The organization recognized by the International Olympic Committee. The term *National Olympic Committee* shall also include the National Sport Confederation in those countries where the National Sport Confederation assumes typical *National Olympic Committee* responsibilities in the anti-doping area.

No Advance Notice: A *Doping Control* which takes place with no advance warning to the *Athlete* and where the *Athlete* is continuously chaperoned from the moment of notification through *Sample* provision.

No Fault or Negligence: The *Athlete's* establishing that he or she did not know or suspect, and could not reasonably have known or suspected even with the exercise of utmost caution, that he or she had *Used* or been administered the *Prohibited Substance* or *Prohibited Method*.

No Significant Fault or Negligence: The *Athlete's* establishing that his or her fault or negligence, when viewed in the totality of the circumstances and taking into account the criteria for *No Fault or Negligence*, was not significant in relationship to the anti-doping rule violation.

Out-of-Competition: Any *Doping Control* which is not *In-Competition*.

Participant: Any *Athlete* or *Athlete Support Personnel*.

Person: A natural *Person* or an organization or other entity.

Possession: The actual, physical possession, or the constructive possession (which shall be found only if the *Person* has exclusive control over the *Prohibited Substance/Method* or the premises in which a *Prohibited Substance/Method* exists); provided, however, that if the *Person* does not have exclusive control over the *Prohibited Substance/Method* or the premises in which a *Prohibited Substance/Method* exists, constructive possession shall only be found if the *Person* knew about the presence of the *Prohibited Substance/Method* and intended to exercise control over it. Provided, however, there shall be no anti-doping rule violation based solely on *possession* if, prior to receiving

Possession Comment: Under this definition, steroids found in an *Athlete's* car would constitute a violation unless the *Athlete* establishes that someone else used the car; in that event, the Anti-Doping Organization must establish that, even though the *Athlete* did not have exclusive control over the car, the *Athlete* knew about the steroids and

intended to have control over the steroids. Similarly, in the example of steroids found in a home medicine cabinet under the joint control of an *Athlete* and spouse, the Anti-Doping Organization must establish that the *Athlete* knew the steroids were in the cabinet and that the *Athlete* intended to exercise control over the steroids.

notification of any kind that the *Person* has committed an anti-doping rule violation, the *Person* has taken concrete action demonstrating that the *Person* no longer intends to have *Possession* and has renounced the *Person's* previous *Possession*.

Prohibited List: The List identifying the *Prohibited Substances* and *Prohibited Methods*.

Prohibited Method: Any method so described on the *Prohibited List*.

Prohibited Substance: Any substance so described on the *Prohibited List*.

Provisional Hearing: For purposes of Article 7.5, an expedited abbreviated hearing occurring prior to a hearing under Article 8 (Right to a Fair Hearing) that provides the *Athlete* with notice and an opportunity to be heard in either written or oral form.

Provisional Suspension: See *Consequences* above.

Publicly Disclose or Publicly Report: To disseminate or distribute information to the general public or persons beyond those persons entitled to earlier notification in accordance with Article 14.

Registered Testing Pool: The pool of top level *Athletes* established separately by each International Federation and National Anti-Doping Organization who are subject to both *In-Competition* and *Out-of-Competition Testing* as part of that International Federation's or Organization's test distribution plan.

Sample Specimen: Any biological material collected for the purposes of *Doping Control*.

Registered Testing Pool Comment: Each International Federation shall clearly define the specific criteria for inclusion of *Athletes* in its Registered Testing Pool. For example, the

criteria could be a specified world ranking cut-off, a specified time standard, membership on a national team, etc.

Signatories: Those entities signing the *Code* and agreeing to comply with the *Code*, including the International Olympic Committee, International Federations, International Paralympic Committee, *National Olympic Committees*, National Paralympic Committees, *Major Event Organizations*, *National Anti-Doping Organizations*, and WADA.

Tampering: Altering for an improper purpose or in an improper way; bringing improper influence to bear; interfering improperly to alter results or prevent normal procedures from occurring.

Target Testing: Selection of *Athletes* for *Testing* where specific *Athletes* or groups of *Athletes* are selected on a non-random basis for *Testing* at a specified time.

Team Sport: A sport in which the substitution of players is permitted during a *Competition*.

Testing: The parts of the *Doping Control* process involving test distribution planning, *Sample* collection, *Sample* handling, and *Sample* transport to the laboratory.

Trafficking: To sell, give, administer, transport, send, deliver or distribute a *Prohibited Substance* or *Prohibited Method* to an *Athlete* either directly or through one or more third parties, but excluding the sale or distribution (by medical personnel or by *Persons* other than an *Athlete's Support Personnel*) of a *Prohibited Substance* for genuine and legal therapeutic purposes.

Use: The application, ingestion, injection or consumption by any means whatsoever of any *Prohibited Substance* or *Prohibited Method*.

WADA: The World Anti-Doping Agency.

The role of measurement uncertainty in doping analysis

Elena Spirito* and Francesco Botrè

Laboratorio Antidoping, Federazione Medico Sportiva Italiana,
Largo Giulio Onesti 1, 00197 Rome RM, Italy

E-mail: antidoping.lab@flashnet.it

*Corresponding author

Abstract: The determination of measurement uncertainty is a critical issue in all fields of experimental science; its importance becomes maximal in the specific case of forensic analytical chemistry (including doping analysis), where uncertainty has not only to be calculated with precision, but it also has to be both small and reliable enough to support effective decision making. This contribution gives a general view of the organisation of the activity of the network of the anti-doping laboratories accredited by the World AntiDoping Agency (WADA), focusing in particular on the current situation related to the determination of measurement uncertainty in doping analysis. Representative examples referring to the procedures followed in the case of threshold substances are also presented and discussed.

Keywords: anabolic steroids; doping analysis; nandrolone; norandrosterone; testosterone; testosterone/epitestosterone ratio; uncertainty.

Reference to this paper should be made as follows: Spirito, E. and Botrè, F. (2005) 'The role of measurement uncertainty in doping analysis', *Int. J. Risk Assessment and Management*, Vol. 5, Nos. 2/3/4, pp.374–386.

Biographical notes: Elena Spirito, Dr Eng in Chemical Engineering, is Quality Manager of the Antidoping Laboratory of Rome, Italy, accredited ISO 17025 and by the World AntiDoping Agency (WADA). Her main field of study is focused on the validation and uncertainty evaluation and assessment of instrumental analytical methods, with experience as a Consultant Engineer in the area of environmental impact of engineering technologies and of quality control for design activities in the field of composite structures production.

Francesco Botrè, Dr in Chemistry and in Pharmaceutical Sciences, post-degree specialisation in experimental pharmacology. He is Professor of pharmacokinetics and of ecology at the University of Rome 'La Sapienza'; Scientific Director of the Antidoping Laboratory of Rome, Italy; and Fellow of the World Association of Antidoping Scientists (WAADS). He is also a Member of several scientific societies and of the editorial board of international scientific journals, and author of over 200 scientific publications, including review articles and books, in the fields of biochemical analysis, physiopharmacology and environmental science.

1 Introduction: the activity and organisation of anti-doping laboratories

The control of the illicit use of performance enhancing substances and methods in sport is carried out by a network of anti-doping laboratories accredited by the World Anti-Doping Agency (WADA), that is the international authority responsible for the anti-doping regulation (World Antidoping Agency, 2004a). In this context, the role of the anti-doping laboratories is to detect the presence, in the athlete's body, of any of the doping substances and/or their metabolites and/or markers of administration whose use is prohibited by WADA itself. All prohibited substances and methods are included in a list that is periodically updated and that, at present, comprises over 200 substances (World Antidoping Agency, 2003).

To carry out an anti-doping test means to select the athlete(s) to be tested, to collect, either 'in competition' or 'out of competition', a suitable amount of biological sample (generally urine, with the use of blood limited to the search of specific parameters), to deliver it to the laboratory under a strict chain of custody, where it is analysed – without any reference to the identity of the athlete(s) – according to internationally recognised methods, to detect the putative use of prohibited substances and methods.

Unlike what happens in other areas of analytical chemistry, laboratories are not involved in the sampling procedures: they do not have any role in the selection of the athletes to be tested, in the collection of the biological samples and in their transport to the laboratory. In other words, the forensic nature of the analytical measurement imposes a strict control on all the different stages of the testing process, including those occurring out of the anti-doping laboratory.

At present, there are 33 laboratories accredited by the WADA in the world (Table 1), analysing 200,000 urine/blood samples per year, with a positivity level oscillating around 2% (see www.wada-ama.org for details).

Table 1 The anti-doping laboratories accredited by the World Anti-Doping Agency (last update: February 2005)

| | |
|----------|---|
| Africa | South Africa (<i>Bloemfontein</i>), Tunisia (<i>Tunis</i>) |
| Americas | Brazil (<i>Rio de Janeiro</i>), Canada (<i>Montreal</i>), Colombia (Bogota), Cuba (<i>La Habana</i>), USA (<i>Los Angeles</i>) |
| Asia | China (<i>Beijing</i>), Korea (<i>Seoul</i>), Japan (<i>Tokyo</i>), Malaysia (<i>Penang</i>), Thailandia (<i>Bangkok</i>) |
| Europe | Austria (<i>Seibersdorf</i>), Belgium (<i>Ghent</i>), Czech Republic (<i>Prague</i>), Finland (<i>Helsinki</i>), France (<i>Paris</i>), Germany (<i>Cologne, Kreischka</i>), Greece (<i>Athens</i>), Italy (<i>Rome</i>), Norway (<i>Oslo</i>), Poland (<i>Warsaw</i>), Portugal (<i>Lisbon</i>), Russian Federation (<i>Moscow</i>), Spain (<i>Barcelona, Madrid</i>), Sweden (<i>Stockholm</i>), Switzerland (<i>Lausanne</i>), Turkey (<i>Ankara</i>), UK (<i>Cambridge, London</i>) |
| Oceania | Australia (<i>Sydney</i>) |

Since it is self-evident that a wrong laboratory result, apart from a merely scientific point of view, can have dramatic consequences on the credibility of the whole anti-doping system, many actions have been undertaken, at different levels, to guarantee the solidity of the experimental data.

In particular:

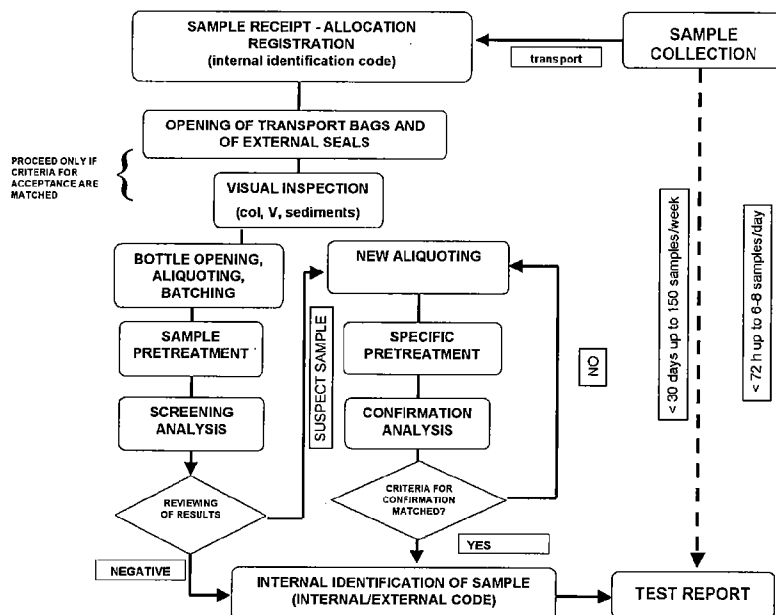
- laboratories have to achieve and maintain ISO 17025 accreditation
- laboratories have to comply with the WADA specific requirements (international standards) (World Antidoping Agency, 2004b)
- samples are sent to the laboratory under a strict chain of custody
- the laboratory is unaware of the identity of the tested athletes
- any adverse analytical finding can be verified by re-analysing a second aliquot of the sample, in the presence of the athlete and of a technical expert.

In the first two points of the above list, and in the correlated technical documents, are included all the requirements the laboratories have to satisfy to gain and maintain the status of a WADA accredited laboratory. More precisely, ISO 17025 accreditation is a prerequisite for all WADA laboratories.

The usual analytical procedure which is followed by all WADA laboratories for all prohibited substances is constituted by two stages: a series preliminary testing (screening), carried out on the whole population of samples to be assayed and the subsequent confirmation analysis, carried out only on those samples which gave positive or even doubtful results after the screening. In the case of threshold substances, laboratories are obliged to follow a validated procedure to assess the degree of confidence of their measurement.

Figure 1 schematically represents the organisation of the internal procedures at the anti-doping laboratory of Rome, Italy.

Figure 1 Block diagram summarising the general organisation of the workflow at the anti-doping laboratory of Rome, Italy



Source: Botrè (2003)

2 Measurement uncertainty and doping analysis

Measurement uncertainty defines a range of values containing the true value. It is self-evident that the determination of measurement uncertainty is a critical issue in all fields of experimental science; its importance becomes maximal in the specific case of forensic analytical chemistry (including doping analysis), where uncertainty has not only to be calculated with precision, but it also has to be both small and reliable enough to support effective decision making.

Historically, the assessment of measurement uncertainty was originally limited to the areas of clinical chemistry and laboratory medicine, pharmaceutical analysis, food and environmental analysis and materials characterisation; recently, inter-laboratory studies aimed at quantifying and improving the reproducibility of measurements have been focused also on forensic assays, commodity testings and biotechnology, including DNA profiling and the control of genetically modified organisms.

Unlike what common sense may suggest, the main job of an anti-doping laboratory is not the protection of the health of an athlete: the aim of the analysis, to maintain the language of forensic science, is 'to supply evidence'. The main differences between 'traditional' clinical chemistry and anti-doping laboratories are highlighted in Table 2.

Table 2 Main differences between clinical and anti-doping tests

Clinical chemistry

- matrices: the most suitable biological material
- aim: diagnostic tests
- test report can be based on:
 - detection and quantitation of diagnostic markers
 - multiparametric profiles
- a doubtful sample can be treated as a positive one (activating further investigation).

Doping control analysis

- matrices: only urine for officially recognised tests
 - aim: to supply evidence
 - test report based on:
 - unambiguous identification of specific drugs/metabolites
 - a doubtful sample is generally treated as a negative one (no further investigation).
-

The logical process to be followed in the determination of measurement uncertainty – including doping analysis – is the following (Ellison et al., 2000): preliminarily, a relationship between the measurand and the parameters upon which it depends has to be defined; secondly, all possible sources of uncertainty have to be identified at all stages of the analytical process; then the specific uncertainty for each one of the above-mentioned stages has to be calculated and converted to standard uncertainties; the latter have to be expressed as variances (to avoid false counterbalancing), then

combined and, finally, the resulting overall uncertainty has to be indicated as 'expanded uncertainty', to ensure the desired confidence level (e.g. 99%).

Anti-doping analysis is a good example of a field where the assessment of measurement uncertainty deserves a dedicated examination. Since none of the anti-doping laboratory methods can be considered a primary method of measurement (i.e. a method having the highest metrological qualities, whose operation can be completely described and understood, for which a complete uncertainty statement can be written down in terms of SI units), it follows that traceability is mandatory.

As stated before, the anti-doping analyses may be performed only by accredited laboratories; this means that they have to be accredited both according to the ISO 17025 and by the World Anti-Doping Agency; more precisely, ISO 17025 accreditation is a fundamental prerequisite to obtain and maintain the status of 'official' laboratory.

It may be significant to specify that, also according to the World Anti-Doping Code International Standard for Laboratories (World Antidoping Agency, 2004b), anti-doping laboratories do not define, in their test reports, a sample as 'positive' or 'negative', but instead report an 'adverse analytical finding'. This definition simply means that a sample was not found to be compliant: this non-compliance may be due to the simple presence of a banned substance, or, in the case of threshold substances, to a value of its concentration exceeding the threshold value itself (Van Eenoo and Delbeke, 2003).

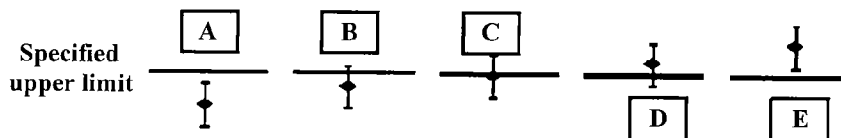
The general expression used, for the determination of combined uncertainty of the variable $y = f(x_i)$ is the following:

$$u(y) = \sqrt{\sum_{i=1}^n \left(\frac{\partial y}{\partial x_i} \right)^2 u^2(x_i)} \quad (1)$$

where it is assumed that there is not any correlated effect among any of the x_i variables.

In practice, to report an adverse analytical finding, it is necessary to identify a prohibited substance, and, in the case of substances with a reporting threshold, to measure a value exceeding the threshold; in the latter case, it is mandatory to express the measurement uncertainty. It is not unusual for a result apparently exceeding the threshold, if taken as a single value or even as a mean value to not be correctly reported as 'above the threshold' if the measurement uncertainty is not taken into account. In Figure 2 it is evident that only case 'E' shows a value that is above the threshold also taking into account the measurement uncertainty.

Figure 2 The role of measurement uncertainty in correctly reporting a value 'above the threshold'



The application of these general principles is here discussed on the basis of the approach followed at the WADA-ISO 17025 anti-doping laboratory of Rome to calculate the measurement uncertainty on two specific procedures:

- the confirmation of nandrolone metabolites, and specifically of norandrosterone, whose urinary concentration threshold is fixed at 2 ng/ml
- the confirmation of an elevated testosterone/epitestosterone (T/E) concentration value, where the threshold has recently been reduced from 6 to 4.

3 Case studies

3.1 Case 1: norandrosterone (nandrolone metabolite): WADA cut off set at 2 ng/ml for a sample of normal density (specific gravity ≤ 1.020)

Following the qualitative identification of norandrosterone (NA), its concentration is measured by GC-MS, on the basis of the area ratio between the signal of NA (ion m/z 405) and that of norandrosterone d4 (NAd4), added as internal standard (ion m/z 409). The ratio NA/NAd4 in the sample is then compared to the same ratio in the positive reference urine, usually spiked with norandrosterone at the threshold value.

For each assayed aliquot, the ratio between the concentration of norandrosterone in the unknown sample (c_{sample}) and the concentration in the positive spiked urine (c_{USP}) is calculated using this formula:

$$Y = \frac{c_{sample}}{C_{USP}} = \frac{R_{ac}}{R_{au}} \quad (2)$$

where

$$R_{ac} = \frac{A_{cNA}}{A_{cNAd4}} \quad (3)$$

i.e. the area ratio between NA and NAd4 in the sample, and

$$R_{au} = \frac{A_{uNA}}{A_{uNAd4}} \quad (4)$$

i.e. the area ratio between NA and NAd4 in the positive reference urine.

In this case, $u(Y)$, that is relative the uncertainty (u), related to the measurement (Y) may be defined as

$$\frac{u(Y)}{Y} = \sqrt{\left(\frac{u_{Rac}}{Rac}\right)^2 + \left(\frac{u_{Rau}}{Rau}\right)^2} \quad (5)$$

where, in turn, Rac and Rau , representing the concentration ratios, are defined as follows:

$$Rac = \frac{x \cdot V_{ur}}{V_{pd} \cdot S_{md}} \quad (6)$$

$$Rau = \frac{S_m \cdot V_p}{V_{pd} \cdot S_{md}} \quad (7)$$

and x is the unknown concentration in the sample, V_{ur} the urine sampled volume, V_{pd} the internal standard sampled volume, S_{md} the internal standard concentration, S_m the standard solution concentration and V_p the standard sampled volume.

R_{ac} and R_{au} uncertainties can now be defined as follows:

$$\frac{u_{R_{ac}}}{R_{ac}} = \sqrt{\left(\frac{u_{V_{ur}}}{V_{ur}}\right)^2 + \left(\frac{u_{S_{md}}}{S_{md}}\right)^2 + \left(\frac{u_{V_{pd}}}{V_{pd}}\right)^2} \quad (8)$$

$$\frac{u_{R_{au}}}{R_{au}} = \sqrt{\left(\frac{u_{V_p}}{V_p}\right)^2 + \left(\frac{u_{S_m}}{S_m}\right)^2 + \left(\frac{u_{S_{md}}}{S_{md}}\right)^2 + \left(\frac{u_{V_{pd}}}{V_{pd}}\right)^2} \quad (9)$$

In addition to the above, we can finally consider other two uncertainty fonts, namely the area ratio repeatability and the method linearity. The composed uncertainty of the ratio is therefore given by:

$$u(Y)_{comp} = \sqrt{u(Y)^2 + u_{rip}^2 + u_{lin}^2} \quad (10)$$

where u_{rip} = areas ratio repeatability uncertainty calculated during the analysis and u_{lin} = uncertainty related to the linearity of the validated method (i.e. residual standard deviation).

3.2 Case 2: norandrosterone (nandrolone metabolite): correction to apply in the case of a sample with elevated density (specific gravity > 1.020)

In the case of samples with a specific gravity higher than 1.020, the NA concentration threshold of 2 ng/ml has to be increased proportionally to the value of the specific gravity. This means that, in this case, a further source of uncertainty is present, deriving from the measurement of the specific gravity of the sample. This contribution may be incorporated in the overall uncertainty as follows: first of all it is necessary to rewrite Equation (2) taking into account this correction:

$$Y^I = \frac{R_{ac}}{R_{au}^I} \quad (11)$$

where

$$R_{au}^I = \frac{(\rho_c - 1)R_{au}}{0.02} \quad (12)$$

so that Equation (11) becomes

$$Y^I = \frac{0.02R_{ac}}{R_{au}(\rho_c - 1)} \quad (13)$$

The overall relative uncertainty (previously given by Equation (5)) is now given by

$$\frac{u(Y^I)}{Y^I} = \sqrt{\left(\frac{u_{R_{ac}}}{R_{ac}}\right)^2 + \left(\frac{u_{R_{au}}}{R_{au}}\right)^2 + \left(\frac{u_{\rho_c}}{\rho_c - 1}\right)^2} \quad (14)$$

where ρ_c is the sample specific gravity and u_{ρ_c} is the uncertainty associated to ρ_c .

Finally, the composed uncertainty of the corrected ratio is given by:

$$u(Y^I)_{comp} = \sqrt{u(Y^I)^2 + u_{rip}^2 + u_{lin}^2} \quad (15)$$

with u_{rip} and u_{lin} defined as in Equation (10).

In the case of a positive reference urine spiked with NA at a final concentration of 2 ng/ml, the concentration of NA in the sample is reported as 'above the threshold' whenever the mean value of Y (or Y^I in the case of a value of the specific gravity > 1.020), subtracted of its expanded uncertainty $U(Y)$ – or $U(Y^I)$ in the case of specific gravity > 1.020 , is still greater than 1, with $U(Y)$ and $U(Y^I)$ defined as follows:

$$u(Y)_{ex} = k \cdot u(Y)_{comp} \quad (16)$$

$$u(Y^I)_{ex} = k \cdot u(Y^I)_{comp} \quad (17)$$

where k defines the coverage factor.

3.3 Case 3: elevated testosterone/epitestosterone concentration ratio

Testosterone is an androgenic anabolic steroid, naturally synthesised by the body and excreted in the urine mainly as glucuronate. A reliable index of synthetic testosterone administration is the value of the concentration ratio between testosterone and its natural epimer, epitestosterone: the value of this ratio is usually around 1 and is, however, quite constant over time for the same subject. An abnormally elevated value of the testosterone/epitestosterone (T/E) concentration ratio is considered sufficient to drive further investigations, these being either longitudinal tests and/or endocrinological investigation (to assess whether the elevated value of the T/E ratio may be traced to physio-pathological causes) and/or advanced physico-chemical tests, e.g. GC-(C)IRMS, gas chromatography coupled to combustion isotopic ratio mass spectrometry.

From a general point of view, testosterone and epitestosterone concentrations in the urine sample are quantified, also in this case by GC-MS; the ion m/z 432 is monitored for both target compounds, eluting at different retention times.

A calibration curve, based on at least three points, is drawn for both T and E; the general equation of this (linear) calibration curve can be written as

$$Y = BX + Q \quad (18)$$

where

$$Y = \frac{A_c}{A_{deut}} \quad (19)$$

is the ratio between the area of the target compound and that of its specific internal standard (usually its deuterated isotope), and

$$X = \frac{x_c}{x_{deut}} \quad (20)$$

is the ratio between the concentration of the target compound and that of its deuterated internal standard.

It follows that the concentration of the target compound in the sample is given by:

$$x_c = \frac{(Y - Q)}{B} x_{deut}. \quad (21)$$

According to the WADA International Standard for Laboratories and the relevant technical documents, the laboratory reports an adverse analytical finding whenever the value of the concentration ratio between testosterone and epitestosterone is greater than 4.

As can be seen, to take into account the measurement uncertainty in this case is more complex than for the norandrosterone concentration, since the concentrations of two different compounds have to be measured in the sample and their values have also to be somehow correlated. In this case, one possible approach would be to determinate the concentrations of T and E separately and then to calculate the corresponding concentration ratio. The first step is to determine the concentration ratio between the target compound (T or E) and its deuterated internal standard. The uncertainty of this value depends on two separate sources: the repeatability uncertainty, u_r , and the calibration curve uncertainty, u_c . These contributions can be linked by the following relation, to give the overall uncertainty, u_X :

$$u_X = \sqrt{u_r^2 + u_c^2}. \quad (22)$$

Let us now consider the two components of the right hand term of the above equation separately.

3.3.1 Repeatability uncertainty U_r

Two cases can be distinguished and considered independently.

Firstly, the validation has demonstrated that there is a significant difference on repeatability between the instrumental injection and aliquot preparation, with the latter larger than the former. In this case the repeatability uncertainty is defined as

$$u_r = \sqrt{\frac{S_{ILT}^2}{h} + \frac{S_r^2}{hk}} \quad (23)$$

where S_r^2 = injection contribution expressed as mean variance of each aliquot during validation process; h = number of aliquots prepared during the analysis; k = number of injection made during the analysis.

It can now be written:

$$S_{ILT} = S_{IMT}^2 - \frac{S_r^2}{n} \quad (24)$$

where, in turn, S_{IMT}^2 = sample preparation contribution expressed as variance of the aliquot means; n = number of injection during validation process.

Secondly, the validation has demonstrated that there is no significant difference on repeatability between instrumental injection and aliquot preparation. In this case the repeatability uncertainty is given by

$$u_r = \frac{S_{rt}}{\sqrt{N}} \quad (25)$$

where S_{ri} is the standard deviation of the validation total number of tests (aliquot and injections) and N is the total number (aliquot \times injections) of tests during the analysis.

3.3.2 Calibration curve uncertainty U_c

This component of the overall uncertainty is more complex to deal with; basically, the following equations apply:

$$s_{x0} = \frac{s_{y/x}}{b} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}} \quad (26)$$

$$s_{y/x} = \sqrt{\frac{\sum_i (y_i - y_{ic})^2}{n - 2}} \quad (27)$$

where, b is the slope of the calibration curve; n is the number of curve points; \bar{y} is the mean value of calibration points; m is the number of reads for each sample; \bar{x} is the mean concentration of the calibration points; $s_{y/x}$ is the residual standard deviation and y_{ic} is the y value calculated using the curve at i point.

The results obtained by using the calibration curve are ratios between compound concentration x_c and internal standard x_{deut} and are given by

$$X = \frac{x_c}{x_{deut}} \quad (28)$$

Now, if, from the above equation, the concentration of the target compound is expressed as $x_c = X \cdot x_{deut}$, the compound concentration uncertainty u_c is given by:

$$u_c = \sqrt{X^2 u_{x_{deut}}^2 + x_{deut}^2 u_X^2} \quad (29)$$

where $u_{x_{deut}}$ is the uncertainty due to internal standard concentration in the sample ($x_{deut} = V_{Smd} * S_{md} / V_{ur}$) expressed as:

$$u_{x_{deut}} = x_{deut} \sqrt{\left(\frac{u_{V_{Smd}}}{V_{Smd}}\right)^2 + \left(\frac{u_{V_{ur}}}{V_{ur}}\right)^2} \quad (30)$$

where, again, S_{md} is the concentration of solution, $u_{V_{Smd}}$ is the uncertainty of the volume of solution sampled and $u_{V_{ur}}$ is the uncertainty of the volume of urine sampled.

3.3.3 Uncertainty on sampled volumes ($U_{V_{Smd}}$ and $U_{V_{ur}}$)

Sampled volumes uncertainty, u_{V_-} , is defined as follows:

$$U_{V_-} = \sqrt{u_r^2 + \left(\frac{u_d}{\sqrt{3}}\right)^2} \quad (31)$$

where u_r is the repeatability uncertainty and u_d is the certified uncertainty due to the glassware used.

3.3.4 Composed uncertainty on the value of the T/E concentration ratio

The contribution due to testosterone concentration and epitestosterone concentration, calculated as expressed above, must be combined using the function $Y = T/E$.

The related uncertainty is given by:

$$u(Y) = Y * \sqrt{\frac{u_T^2}{T^2} + \frac{u_E^2}{E^2}} \quad (32)$$

where u_T is the uncertainty on testosterone concentration (T) and u_E is the uncertainty on epitestosterone concentration (E).

3.3.5 Expanded uncertainty

Also in this case the expanded uncertainty is dependent on the chosen coverage factor k :

$$U_e = k u_e. \quad (33)$$

4 Conclusions

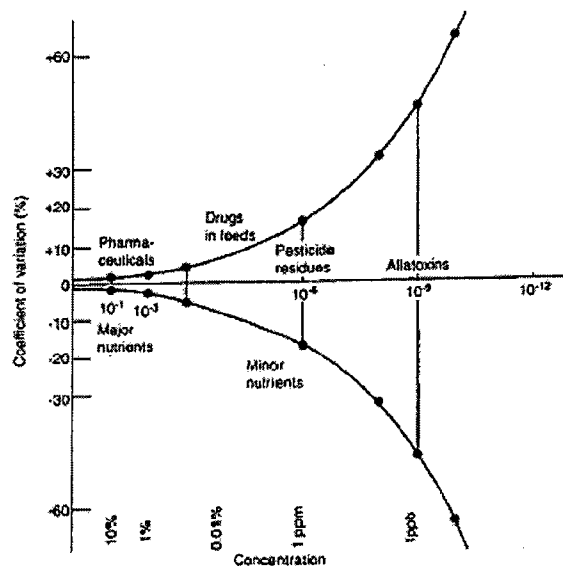
It may be interesting to compare the representative cases presented above with a generally applicable approach to the determination of relative uncertainty. Perhaps the most general relationship which correlates the measurement uncertainty (expressed as RSDR) to the expected concentration, C , of the analyte in the sample is given by the Horwitz equation (Horwitz et al., 1980):

$$\text{RSDR} = 2C^{-0.1505}. \quad (34)$$

The above relation has been drawn following a statistically significant number of inter-laboratory comparative studies that, although not involving the network of the accredited anti-doping laboratories, may be considered applicable also in this context.

The Horwitz function is often proposed in its graphical form: for indeed, plotting Equation (34) generates the so called 'Horwitz trumpet', reproduced in Figure 3.

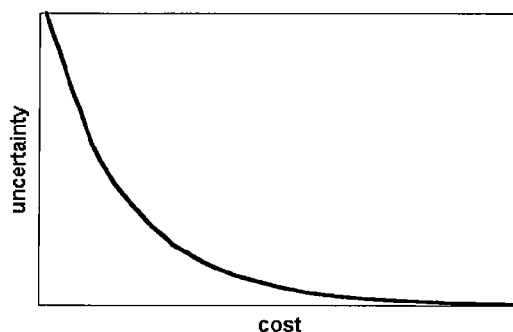
Figure 3 The 'Horwitz trumpet' (plotted on the basis of Equation (34)), correlating the relative uncertainty (expressed as coefficient of variation %) to the expected concentration of analyte in the sample (Horwitz et al., 1980)



It may be interesting to stress that most of the anti-doping cases (and particularly the confirmation of nandrolone metabolites) lie in the aflatoxins-range ($2 \text{ ng/ml} = 2 \text{ ppb}$ is indeed the threshold value of norandrosterone, the main metabolite of nandrolone), characterised by an estimated uncertainty of roughly 40–45%. According to the data represented above, confirmed also by other studies (Van Eenoo and Delbeke, 2003), the uncertainty of the anti-doping laboratories closely match with the Horwitz figures, being in most cases even lower than that expected.

A final comment concerns some economical considerations: it is self-evident that a thoughtful consideration of measurement uncertainty is a critical issues in all areas of analytical chemistry, especially whenever the experimental results are discussed on forensic grounds. However, it must be understood that precision is not cheap: as a general trend, the uncertainty associated with a laboratory method is inversely proportional to the cost of the method itself (Figure 4). In general, technological innovation, professional expertise and technical skill can drive a more or less pronounced shift of the curve towards the origin, but nonetheless the general trend is qualitatively the same for all methods.

Figure 4 The inversely proportional relationship that defines that increasing economical resources are necessary to reduce the uncertainty of measurement



In this sense, the recent reorganisation of anti-doping regulations given by the WADA and, at the same time, the activity of the network of the accredited laboratories, carried out mainly by the World Association of Anti-Doping Scientists (WAADS) ensure that all methods followed by the laboratories are 'fit to purpose', the latter being to provide 'clear and convincing' evidence of any detectable doping offence based on an adverse analytical finding.

References

- Botrè, F. (2003) 'Drugs of abuse and abuse of drugs in sportsmen: the role of in vitro models to study effects and mechanisms', *Toxicology in Vitro*, Vol. 17, pp.509–513.
- Ellison, S.L.R., Rosslein, M. and Williams, A. (Eds) (2000) *Quantifying Uncertainty in Analytical Measurement*, Eurachem/Citac Guide CG4.
- Horwitz, W., Kamps, L.R. and Boyer, K.W. (1980) 'Quality assurance in the analysis of foods for trace constituents', *J. Assoc. Off. Anal. Chem.*, Vol. 63, pp.1344–1354.

Van Eenoo, P. and Delbeke, F.T. (2003) 'Letter to the editor. Reply to "Measurement uncertainty and doping control in sport"', by A. van der Veen (Ed) *Accred. Qual. Assur.*, Vol. 8, pp.477-479.

World Antidoping Agency (2003) *The World Antidoping Code*, Montreal, Canada.

World Antidoping Agency (2004a) *The Prohibited List International Standard*, Montreal, Canada.

World Antidoping Agency (2004b) *The WADA International Standard for Laboratories*, Montreal, Canada.

Appendix D: Error List

This is a list of apparently obvious documentation errors: record keeping errors, simple math errors, or obvious errors in procedure. This list does not address scientific principles or the validity of conclusions. Many of the pages in the document package are boiler-plate machine printed protocols. Most of the pages listed below are handwritten errors.

| Page | ISO Violated? | Errors | Issues | Comments |
|--------------------|---------------|--------|--------------------------------------|--|
| USADA Notice | | 2 | Type Lab | The notice for 995474 is listed as "Out of Competition" The WADA accredited lab is listed as "University of California at Los Angeles" |
| USADA0004 | | 2 | Sample ID Calculation | A partial lab identification number or sample number has been crossed out without initial or date. Specific gravity calculation not based on 0.8 (1.020 vs. 1.025). |
| USADA0007 | | 2 | Cross-out Value | Cross-out on last line. Specific gravity is listed here as 1.026. Everywhere else, it is listed as 1.025. |
| USADA0008 | Yes | 1 | Sample ID | In the middle of the page, second column, a sample is listed as 995475. Floyd's 17th stage number was 955474. This page is missing in the AFLD filing. This may be an effort to cover-up this error and malfeasance. |
| USADA0009 | Yes | 1 | | There has been a correction of number from 992474 to 995474. |
| USADA0024 | Yes | 3 | Sample ID Time/date Cross-outs | Floyd's sample/sample number is not properly recorded as having been transported. Handwriting legibility: a 6 vs. a 4? The LNDD reception time is 9h35. It should be listed as 21h35 or 9:35 PM. There is an overwriting of blood specimen 149986. There is a cross out in the bottom left signature. |
| USADA0027 →0028 | ? | 1 | No page | Page 23 in the LNDD numbering system is missing. |
| USADA0043 | Yes | 5 | Cross-outs | 2 cross-outs. |

| | | | | |
|-----------|-----|---|----------------------------|--|
| USADA0044 | Yes | 4 | Cross-outs | 2 cross-outs. |
| USADA0057 | Yes | 1 | Cross-out | The date is overwritten in the AFLD version. |
| USADA0058 | | 1 | Custody | No record of this work in chain of custody |
| USADA0079 | Yes | 6 | Cross-outs Ref solution | 3 cross-outs. One of lab sample number, two cross outs of initial of lab operator. At least 3 wrong reference solution(s) for epitestosterone and for testosterone, probably more. |
| USADA0081 | | 1 | Calculation | Specific gravity calculation not based on 0.8 (1.020 vs. 1.025). |
| USADA0101 | | 1 | Extra page | This page, present in the USADA numbering system, in an extra page in the LNDD numbering system between 74 and 75 |
| USADA0083 | | 1 | Extra page | This page, present in the USADA numbering system, in an extra page in the LNDD numbering system between 75 and 76 |
| USADA0086 | | 1 | Extra page | This page, present in the USADA numbering system, in an extra page in the LNDD numbering system between 77 and 78 |
| USADA0120 | | 1 | Custody | No record of overnight storage. |
| USADA0149 | | ? | Sample ID | According to the record, sample f2b was used in Fraction F2. I see no explanation why this was done. There is no record of this subtraction in the chain of custody. |
| USADA0200 | Yes | 8 | Cross-outs Sample | 6 cross-outs. Orphaned notation. At least one reference solution errors for epitestosterone. Probable reference solution errors for testosterone (two numbers for reference solution with same concentration). |
| USADA0223 | | 1 | Calculation | Specific gravity calculation not based on 0.8 (1.020 vs. 1.025). |

| Page | ISO/WADA Violated? | Errors | Issues | Comments |
|------------|--------------------|--------|--------------------------------------|--|
| 'B' sample | | | | |
| USADA0229 | Yes | 3 | Sample ID Time/date Cross-outs | Floyd's sample/sample number is not properly recorded as having been transported. Handwriting legibility: a 6 vs. a 4? The LNDD reception time is 9h35. It should be listed as 21h35 or 9:35 PM. There is an overwriting of blood specimen 149986. |

| | | | | |
|-----------|-----|---|--|--|
| | | | | There is a cross out in the bottom left signature. |
| USADA0252 | ? | 1 | Custody | Opening of sample container. The method used by USCLA is that the person who opens the container signs—not the whole staff present. There is no record as to who was responsible for physically performing the procedure. |
| USADA0257 | Yes | 2 | Custody | The chain of custody records that the 'B' sample IRMS was performed on August 3, 2006. There is no record of an analysis performed on August 3, 2006. There is no chain of custody documenting the work performed on August 4, 2006. |
| USADA0260 | Yes | 1 | Calculation | Specific gravity calculation not based on 0.8 (1.020 vs. 1.025). |
| USADA0288 | Yes | 3 | Sample ID Cross-outs Calculation | Sample number (Echantillon: 478/07 994474.) Floyd was 995474. The lab identification number is wrong. It is reported as 478/07. It should be 178/07. The AFLD document corrects this, improperly—and suggests malfeasance. Specific gravity calculation not based on 0.8 (1.020 vs. 1.025). |
| USADA0300 | | 1 | Custody | No record of overnight storage. |
| USADA0309 | | 1 | Custody | No record of this work, and following pages in this section, in chain of custody. |
| USADA0323 | | 1 | Sample ID | According to the record, sample f3 was used in Fraction F2. There was a similar problem in the 'A' sample, USDA0149. The corresponding trace graphs are too small to be legible. Analyzing the wrong aliquot fraction cannot be ruled out. |
| USADA0352 | | 2 | Calculation | The report erroneously reports androsterone as (positive) 3.51 rather than (the negative) -3.51. The conclusion states that two metabolites were abnormal. Androsterone is wrongly reported as abnormal. Its value is not beyond the WADA's minimum positivity criteria (3.00‰) plus error range (0.8‰). This is clearly explained in Spirito. ¹⁴⁴ |

| Page | ISO/WADA Violated? | Errors | Issues | Comments |
|--------------------|--------------------|--------|--------|--------------|
| Reference Solution | | 15 | | Probably 15. |
| | | | | |

¹⁴⁴ Spirito, E. et al. The role of measurement uncertainty in doping analysis. *Int. J Risk Assessment and Management*. 5 (2/3/4), 374-386. (2005).

| Page | ISO/WADA Violated? | Errors | Issues | Comments |
|---------------------|--------------------|--------|------------------|--------------------------|
| Longitudinal Report | | 1 | Date error | Date error for stage 15. |
| | | | T/E ratio errors | Working on. At least 4. |

| Page | ISO/WADA Violated? | Errors | Issues | Comments |
|---------------------|--------------------|--------|-------------|--|
| Whistle blower Docs | ? | | Contaminant | Lack of control verification procedure, implemented 8 months after Tour de France 2006. For discussion, see page 151. |

Table 28. Documentation error list.

BEFORE THE AMERICAN ARBITRATION ASSOCIATION
North American Court of Arbitration for Sport Panel

United States Anti-Doping Agency,

Claimant,

v.

Floyd Landis,

Respondent.

**DECLARATION OF SIMON
DAVIS**

AAA No. 30 190 00847 06

I, Simon Davis, declare:

1. I am the Technical Director of MS Solutions. I make this declaration based on my personal knowledge, and if called as a witness, I could and would testify competently to the matters set forth herein.
2. I received a Bachelor of Science Honours in Environmental Biology from Oxford Brookes University in 1991. I received a Ph.D. in Stable Isotope Mass Spectrometry from Liverpool JMU, in association with Cambridge University, in 1996.
3. I was a Stable Isotope Systems Engineer for Micromass UK Ltd. from May 1997 to July 1998. From July 1998 to June 2000, I was a Staff Scientist at the Lawrence Berkley National Laboratory, Berkley University. I rejoined Micromass UK Ltd. as a Development Project Leader for the Inorganic MS Group from June 2000 to March 2003. From March 2003 to March 2005, I was a Research Officer for Queens University. In March 2005, I joined MS Solutions as the Technical Director.

4. Based on my education and experience, I am familiar with the testing commonly used in anti-doping detection: The Testosterone to Epitestosterone ratio test ("T/E test") and the Carbon Isotope Ratio test ("CIR").
5. The T/E test is performed using a Gas Chromatography/Mass Spectrometer ("GC/MS") instrument, which calculates the ratio of testosterone to epitestosterone in a sample. The T/E test calculates this ratio by comparing the specific ion signal – called a "response" – of testosterone to the response of epitestosterone.
6. The GC/MS cannot distinguish between synthetic and natural testosterone, however, because their mass spectra are identical. Epitestosterone is an inert epimer of testosterone, which means that it has the same chemical makeup as testosterone, but because of a difference in chirality (a geometric difference – sort of a mirror image) at one location, epitestosterone is not biologically active.
7. In theory, the ratio of testosterone to epitestosterone in urine in adult males should be approximately 1:1. In fact, however, ratios as high as 15:1 or higher could be normal; conversely, some individuals naturally have low urinary T/E ratios that do not change even with the administration of exogenous testosterone.
8. I am also familiar with the CIR, which also has been described as the test for "synthetic testosterone." Generally, molecules are composed of atoms; specifically, biological molecules are composed primarily of Carbon, Oxygen and Hydrogen atoms. Carbon, in its basic form, is an atom composed of six electrons, six protons and six neutrons. In nature, however, most atoms have one or more stable isotopes. A stable isotope is an atom that has "extra" neutrons. In the case of carbon, ^{13}C is a stable isotope that has one "extra" neutron. The actual carbon isotope makeup of any

individual will vary based on his or her diet. The same is true for plants. As it turns out, the particular plants (mostly soy) used for the building blocks of synthetic or pharmaceutical testosterone are particularly low in ^{13}C , especially when compared to the levels found in most humans.

9. The CIR test is performed using an IRMS instrument, which measures the ratio of $^{13}\text{C}/^{12}\text{C}$ in a target analyte. The theory behind the test is that synthetic testosterone, which is usually soy-based, will be depleted in carbon 13. To account for individual variabilities in things such as diet, which can affect the $^{13}\text{C}/^{12}\text{C}$ ratio, the test compares the $^{13}\text{C}/^{12}\text{C}$ ratio of a testosterone metabolite that is believed to be affected by exogenous testosterone to the $^{13}\text{C}/^{12}\text{C}$ ratio of an endogenous reference compound that is believed not to be affected by exogenous testosterone. By comparing the difference in the $^{13}\text{C}/^{12}\text{C}$ ratio between a testosterone metabolite and a testosterone precursor, or other endogenous reference compound, the CIR test can determine the likelihood of testosterone being from an exogenous source.
10. In theory, for any individual at any one time the $^{13}\text{C}/^{12}\text{C}$ ratio of a testosterone precursor should be identical (or very close) to that of a testosterone metabolite. If a person is using exogenous testosterone, however, there will be a detectable and significant difference between the $^{13}\text{C}/^{12}\text{C}$ ratio in a testosterone metabolite and a testosterone precursor. In other words, if a person is taking exogenous testosterone, his or her $^{13}\text{C}/^{12}\text{C}$ ratio for a testosterone metabolite will be different than the ratio for a testosterone precursor.

11. Once the $^{13}\text{C}/^{12}\text{C}$ ratio, commonly reported in the delta per mil notation ($\delta^{13}\text{C}\text{‰}$), for the testosterone metabolites is calculated, the ratio can be compared with the positivity criteria mandated by the World Anti-Doping Agency.

12. In conjunction with this case, I have reviewed Laboratoire National de Depistage du Dopage's (LNDD) laboratory document package concerning Floyd Landis. I have also reviewed the United States Anti-Doping Agency's responses to Mr. Landis's Second Request for Production.

13. I have relied upon the documents referenced above, my education, my experience, and generally accepted principles within the scientific community in reaching the following conclusions:

- a. The Optima GC 1.67-2 software was originally written for the Micromass Optima IRMS and not the Isoprime IRMS instrument. This software is now 10 years old and can be identified by its code number 1.67-2.
- b. Since version 1.67-2 of software was produced, there have been six major version releases of software for the Isoprime. These include (1) Version 1.67-3 (OS2 operating system) (2) Version 1.67-4 (OS2 operating system), (3) Masslynx Version 3.5i (Windows NT), (4) Masslynx Version 3.6i (Windows NT), (5) Masslynx Version 4.0 (Windows XP) and (6) Ion Vantage Version 1.0 (Windows XP).
- c. The newer versions of the software have the following improvements:
 - i. The newer software includes a new set of electronics with a new set of firmware for the systems head amplifier that corrected errors in the OS2 head amplifier firmware.

- ii. The newer software has the ability to control the GC portion of the GC-C-IRMS, whereas in the OS2 versions of the software, the operator has to manually program the GC.
- iii. The newer software traces any changes that are made to the data post acquisition. For instance, if the software is reprocessed with different integration parameters this would be recorded in all Masslynx and Ion vantage systems, but not in any OS2 systems.
- iv. The newer software contains a standards library, for the automated storage and retrieval of standards values and data. OS2 requires the standards to be applied manually post acquisition.
- v. The newer software has fully documented and tested background subtraction routines. The method and validity of the background routines in the OS2 software is unknown and undocumented. All documentation of the OS2 routines was lost when Micromass purchased Isotech (the developers of the original software).
- vi. The newer software has improved peak detection – the true nature of the OS2 detection methods is unknown as no documentation remains as to the method used.
- vii. The newer software provides “read-backs” that allow the true state of the Isoprime to be observed and recorded. The OS2 system offers no read backs.
- viii. The newer software works on a modern operating system for which you can obtain up-to-date anti-virus and malware software. OS2 Warp

(the latest software that version 1.67-2 will run on) is no longer supported by IBM and no anti-virus or security software is available.

ix. The newer software is compatible with a number of Laboratory Integrated Management Systems. This is used for the control of results management.

d. On account of the age of the software, and the fact that it was not designed for this specific IRMS instrument, there is a serious question about whether it is capable of delivering consistently accurate results.

e. If the new software were used, it would provide better peak detection, tested and documented background subtraction routines and would remove any errors in the head amplifier firmware. It would also provide a stable and modern operating system with up to date anti-virus and other security software.

f. Production of the Electronic Data Files ("EDFs") is necessary for the fair adjudication of this matter because the EDFs will assist Mr. Landis' experts in determining whether errors in reporting the results may have stemmed from:

- i. Errors in the Isoprime head amplifier software.
- ii. Incorrect and wrongly applied background subtraction.
- iii. Poor peak integration.
- iv. Inappropriate reprocessing of the original data files.

g. Moreover, if the EDFs are provided, it will be possible to identify if the data reported by the laboratory has been reprocessed. Although the OS2 software does not provide a traceable audit of any reprocessing, it is possible to extract

the original raw data which can then be compared to those results reported by the LNDD.

14. As to the use and maintenance of a GC-IRMS instrument, I have consulted my copy of the Isoprime User Manual, which I have given to Mr. Landis and his counsel. The Isoprime User Manual states that the operating pressure for the GC-IRMS instrument is between 2 and 4E-6 mbar.

15. I have also reviewed USADA 0176 and confirmed that it shows that the GC-IRMS instrument was operating at a pressure of 5.2E-6.

16. I have relied upon the Isoprime User Manual and USADA 0176, my education, my experience, and generally accepted principles within the scientific community in reaching the following conclusions:

- a. Operating the GC-IRMS instrument at pressures of 5E-6 millibars or above can result in reduced sensitivity and precision of the reported results and increased variance values. All mass spectrometers require a vacuum in order to operate properly, which involves ensuring that the ion beam can pass from the source to the detector system in a manner consistent with the manufacturer's specifications.
- b. Failure to operate this machine properly can result in (1) false detection of atmosphere gas as analyte gas and (2) competitive ionization that results in the reduction of the sensitivity of the instrument to analyte gas, among others problems.

- c. Additionally, the increased pressure recorded in the Penning gauge possibly resulted from increased helium pressure in the GC-C system, causing similar defects in results.
- d. The increased pressure will result in a decreased lifespan of the source filament, which also may increase the reported measure of variance.

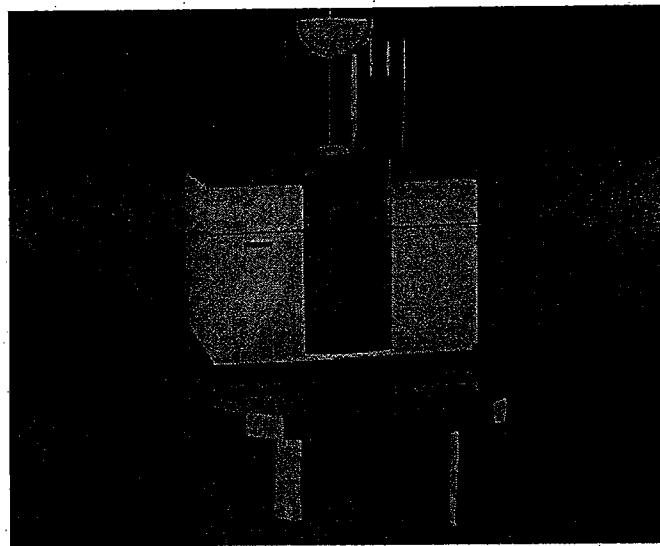
I declare under penalty of perjury under the laws of the State of California that the foregoing is true and correct and that this declaration was executed on February __, 2007 in 13th of February



Simon Davis

IsoPrime EA User Manual
Code No 6666588
Issue 1a

Section 1



Introduction



GDC0522

IsoPrime EA User Manual

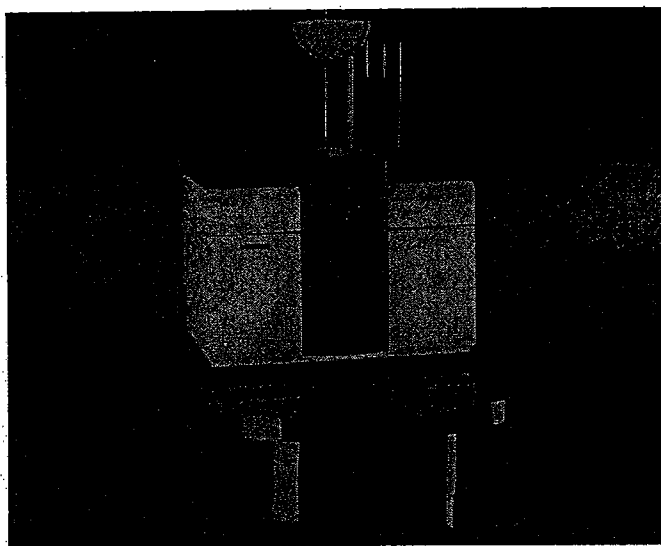
IsoPrime-EA User Manual

This manual provides the operation and maintenance instructions for the IsoPrime-EA. This will include details on the IsoPrime mass spectrometer with the elemental analyser and interface. We have endeavoured to include all operational aspects within this manual, however if you feel more information is required or that you can add some more details, then please contact the Customer Service Department at Micromass UK Ltd. or your local Micromass representative.

IsoPrime EA User Manual

IsoPrime EA User Manual
Code No 6666588
Issue 1a

Section 2



Safety Notices

IsoPrime-EA User Manual

Warranty

The information contained within this document is subject to change without notice.

Micromass UK Ltd. makes no warranty of any kind with regard to this material, including, but not limited to, the implied warranties of merchantability and fitness for a particular purpose.

Micromass UK Ltd. shall not be liable for errors contained herein or for incidental damages in connection with the furnishing, performance, or use of this material.

Safety information

The IsoPrime mass spectrometer meets the following IEC (International Electrotechnical Commission) classifications.

Safety Class 1

Transient Overvoltage Category II

Pollution Degree 2

This unit has been designed and tested in accordance with recognised safety standards and designed for use indoors.

If the instrument is used in a manner not specified by the manufacturer, the protection provided by the instrument may be impaired.

Whenever the safety protection of the IsoPrime has been compromised, disconnect the instrument from all power sources and secure the unit against unintended operation.

Suitably qualified personnel only should perform maintenance procedures.

Substituting parts or performing any unauthorised modification to the instrument may result in a safety hazard.

Disconnect the mains supply before removing covers.

You must comply with all local and national requirements for electrical and mechanical safety.

Please contact the Customer Service Department at:

Micromass UK Ltd.
Floats road,
Wythenshawe,
Manchester,
M239LZ,
UK

Or your local Micromass representative should you require any further information.

Safety symbols

Warnings in the manual or on the instrument must be observed during all phases of service, repair, installation and operation of this instrument. Failure to comply with these precautions violates the safety standards of design and the intended use of the instrument.

Micromass UK Ltd. assumes no liability for the customer's failure to comply with these requirements.

The following safety notices and symbols are used in the manual or on the instrument.

WARNING

Warnings are given to highlight situations or conditions where failure to observe the instruction could result in injury or death to persons.

CAUTION

Cautions are given to highlight situations or conditions where failure to observe the instruction could result in damage to the equipment, associated equipment or process.



This symbol indicates that there are accompanying instructions which should be referred to for more information.



This symbol indicates where hazardous voltages may be present.



This symbol indicates a hot surface.



This symbol indicates a risk of corrosion.

Site requirements

The information contained within this section will allow the user to ensure that the laboratory is capable of supporting the operation of the IsoPrime mass spectrometer.

Temperature and humidity ranges

This equipment is designed for indoor use only. Operation within the recommended ranges will ensure optimum performance and lifetime.

After exposure to extremes of humidity or temperature, allow 15 minutes for it to return to the recommended ranges.

Temperature

Recommended temperature range: 20-27°C

Safe temperature range: 5-40°C

Humidity

Recommended humidity range: 50-60%

Safe humidity range:

Up to 31°C max. 80%

Then decreasing linearly to max. 50% at 40°C

Altitude

Altitude range: Up to 2000m

Cooling requirements

The IsoPrime is cooled by two cooling fans situated on the rear of the cubicle. Air is drawn in by the right-hand fan (viewed from the front) and expelled from the left-hand fan. Do not obstruct the flow of air around the cooling fans.



Caution: For proper cooling and general safety, always operate the instrument with cover panels properly installed and doors closed.

The rotary pump requires adequate ventilation. Consult the manufacturers instructions for details of the positioning requirements of the rotary pump.

Venting toxic or noxious gasses

The IsoPrime system has two points at which gas may be vented into the atmosphere. The rotary pump exhaust and the reference gas box.

All gas which enters the mass spectrometer will leave via the rotary pump exhaust. The exhaust ports of the rotary pumps are fitted with oil mist filters, however you may wish to connect to an exhaust line (15mm internal diameter) leading to a suitable exhaust point. Consult the rotary pump manufacturers manual for details of exhaust requirements.

The safe exhausting of these fumes should be given careful consideration when working with hazardous materials, for example SO₂.

IsoPrime-EA User Manual

The reference gas box freely vents both the carrier gas (Helium) and the reference gas in use, into the atmosphere.



Caution: If a reference gas injector is to be used for hazardous gasses the user should be aware that the gas is exhausted into the atmosphere both within the reference gas injection box and also from the rotary pump exhaust. It is essential that suitable extraction be installed so that the gas concentration in laboratory air complies with the local Health and Safety standards.

Benchtop requirements

The IsoPrime is a bench top instrument and may be placed on normal laboratory benches. The bench must be of a construction adequate to support the weight of the IsoPrime and associated prep systems. The bench should also be free of any vibration or movement that may impair the performance of the IsoPrime.

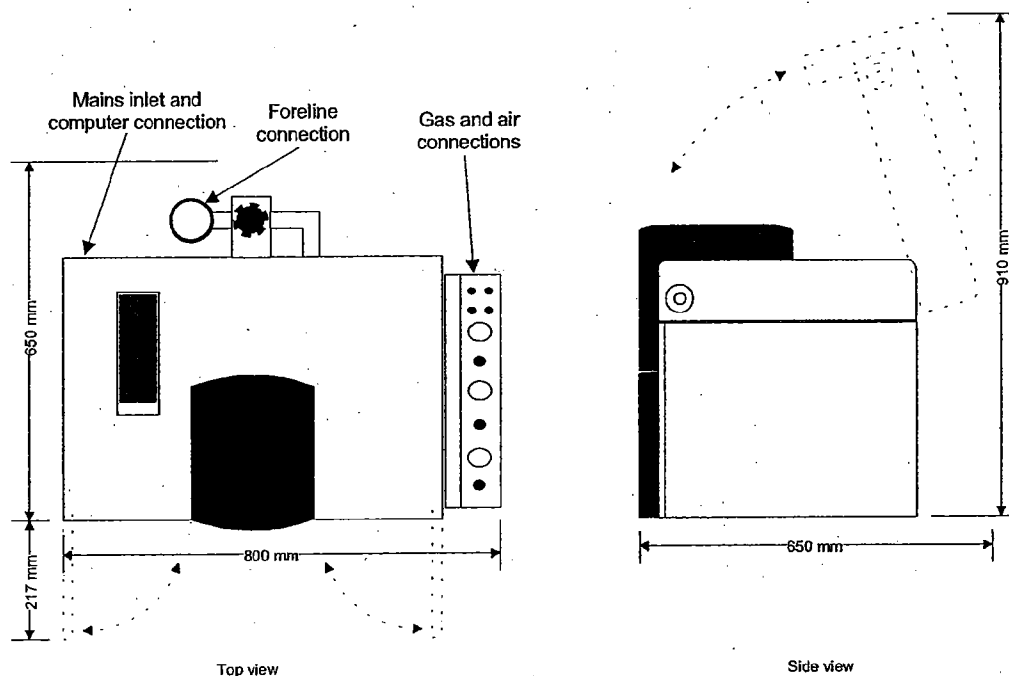
The IsoPrime (including reference gas injector box) has overall dimensions of:

Width 795mm

Depth 585mm

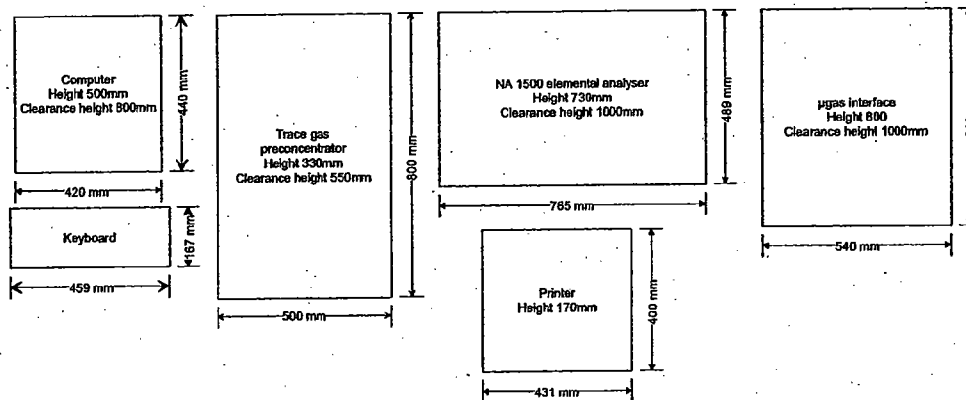
Height 535mm.

The minimum space requirements for the IsoPrime when placed on a bench are detailed in the drawing below. In addition to the clearance indicated there must be a 50mm diameter hole to allow the foreline to pass through or behind the bench. The IsoPrime is supplied with 2m of flexible tubing to connect to the rotary pump, which should be placed on the floor beneath the instrument.



IsoPrime-EA User Manual

The IsoPrime preparation systems require additional bench space as indicated in the following diagram. The preparation systems are normally located on the right hand side of the IsoPrime, and the computer system to the left. Under certain circumstances this positioning may be altered, please contact the factory for advice on where this is possible.



The GC interface is fitted to a freestanding bench and customers may wish to order a freestanding bench for the IsoPrime when ordering a GC interface.

The clearance heights marked on the drawing are for guidance only, shelves or overhanging obstructions will limit access to the instrument and may interfere with cooling.

Allow 100mm between and behind instruments to allow for proper ventilation and access for connections.

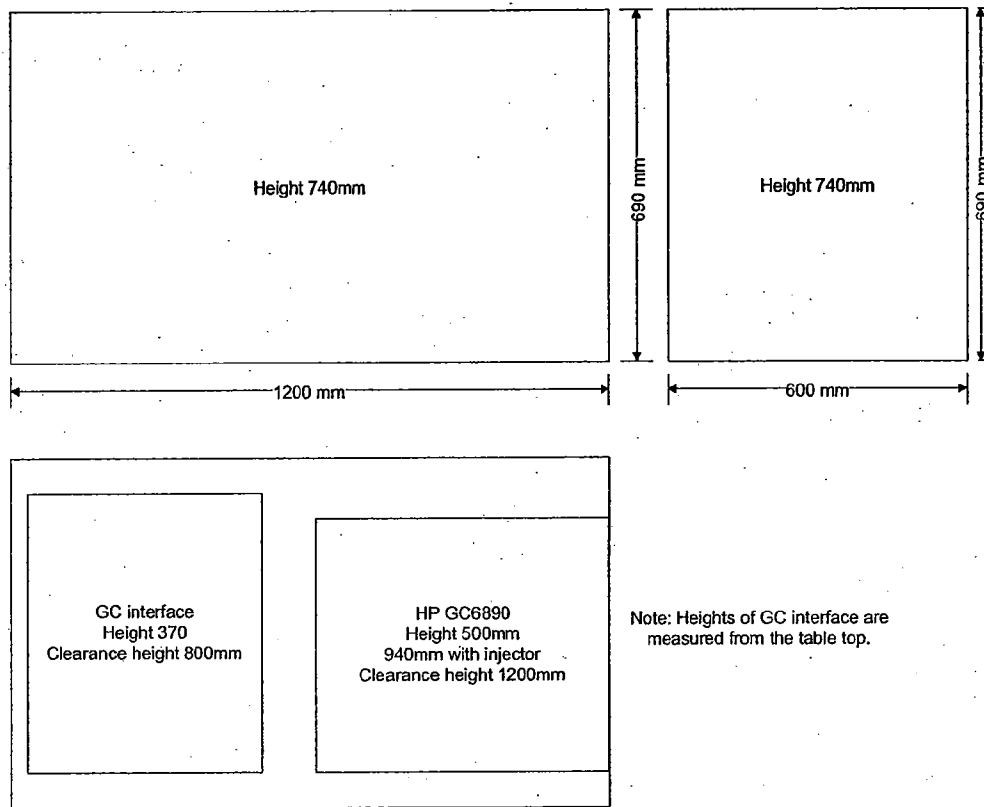
The approximate weights of individual units of the instrument are shown below:

| | |
|-------------------------------------|-------|
| Micromass IsoPrime cubicle | 82kg |
| Permanent magnet | 21kg |
| Electromagnet | 45kg |
| Computer | 55kg |
| Rotary pump | 23kg |
| Micromass GC interface | 130kg |
| Micromass µG interface | 43kg |
| Micromass EA interface | 130kg |
| Micromass Trace gas preconcentrator | 33kg |
| 600mm wide table | 22kg |
| 1200mm wide table | 33kg |

Freestanding requirements

The plan below shows the table sizes available for free standing placement of the IsoPrime and its interfaces. The smaller of the two tables will take the μ gas interface and the Trace gas preconcentrator. The larger table may be used for IsoPrime /reference gas box, elemental analyser or computer and printer. The GC interface is fitted to the larger of the two tables as standard and is shown in the drawing below.

Please allow clear floor space for at least 1 metre surrounding the instrument for maintenance access. The floor must be flat and free from vibration and have sufficient load bearing capacity.



Electrical requirements

The mass spectrometer and preparation systems may each require one or more connections to the mains supply, which should meet the following requirements:

Grounding

A proper earth ground is required for the safe operation of this equipment.

To protect users, the metal cubicle and analyser are grounded through the three-conductor power lead in accordance with the requirements of the IEC.

Earth grounding of the instrument is provided through the three-conductor power lead. When connected to a properly grounded receptacle, the instrument is also grounded and minimises shock hazard. A properly grounded receptacle is one that is connected to a suitable earth ground. Proper grounding should be verified.

Line voltage

Preparation systems require a 230V AC, 50-60Hz, single phase supply.

The IsoPrime mass spectrometer and rotary pump require a 110/230V AC, 50-60Hz single phase supply.

One terminal (the neutral) **must** be at earth (ground) potential. This is to ensure that the voltage on all conductors with respect to ground does not rise to an unsafe voltage.

Each circuit is supplied with one or more suitable mains supply cables. Connectors for attaching these cables to the local supply should be provided by the user.

Each circuit is supplied with a 2m cable to attach to the site mains supply. The cables supplied are terminated with a 10A IEC320/C13 cold condition free socket at one end and a UK mains 13A BS1363A plug at the other end. Customers requiring alternative terminations are advised to supply their own leads terminated with a 10A IEC320/C13 cold condition free socket.

Note: The elemental analyser mains cable is supplied with a 2m cable terminated with a UK mains 13A BS1363A plug at one end and an IEC320/C19 16amp connector at the other.

The individual current requirements of the mass spectrometer and preparation systems are shown in the table below. Some circuits are susceptible to large voltage transients. These should be protected by a suitable transient suppressor if the local supply is of poor quality. Some sites may also wish to protect some (the pumping circuits, for example) or all circuits from power failure with an un-interruptable power supply unit. Once again this depends on the quality of the local supply. If you need further advice on your local supply please contact your local Micromass sales and service organisation.

Users should ensure that the provision of the mains supplies described in this document is in accordance with any local regulations.

IsoPrime-EA User Manual

| System | Circuit | Source | Max. current @ 110V 60Hz | Max. current @ 230V 50Hz | Max. Fuse Rating | Approx. Heat Output | Transient Sensitive |
|----------------|-------------------------|--------|-----------------------------------|-----------------------------------|------------------------|---------------------------|------------------------|
| IsoPrime | Mass spectrometer | Mains | 7.2A | 3.4A | 10AT | 600W | Yes |
| IsoPrime | Rotary pump | Mains | 4.4A | 2.4A | 10AT | 300W | No |
| Data system | Computer + printer | Mains | N/A | 3A | 10AT | 500W | Yes |
| GC interface | Gas Chromatograph | Mains | N/A | 13A | 13AT | 2950W | No |
| GC interface | Interface (Furnace) | Mains | N/A | 3A | 3AT | 300W | No |
| µgas interface | Interface + autosampler | Mains | N/A | 1.2A | 3AT | 130W | Yes |
| EA | Elemental analyser | Mains | N/A | 6A | 13AT | 200W | No |
| Trace gas | Trace gas | Mains | N/A | 2A | 3.15AT | 725W | No |

Note: Each line in the above table represents an individual mains connection and uses an individual mains lead.

Note: mains supply voltage fluctuations should not exceed $\pm 10\%$ of the nominal voltage.

Special gasses

Compressed air

The Micromass IsoPrime requires no compressed air but each of its prep systems requires a regulated compressed air supply of 60 psi. If a compressed air line is not available, a compressor must be supplied.

The air supply should be oil, water and particulate free.

The air-line connections on the reference gas boxes and prep systems are 6 mm (OD) push-fit.

Gas requirements

The IsoPrime mass spectrometer is sensitive to contamination of supply gasses. The presence of any hydrocarbon, water or particulates will have a significant effect upon instrument performance and reliability.

The table below lists the minimum acceptable purity of gasses for use with the IsoPrime.

Not all prep systems require all gasses

| Gas | Purity | O ₂ content | H ₂ O content | Required for |
|-----------------|-----------------------------|------------------------|--------------------------|--------------|
| Helium | 99.999% | | < 1 v.p.m. | All systems |
| Oxygen | 99.998% | | | EA, GC(N) |
| Hydrogen | 99.999% | | < 2 v.p.m. | GC |
| Dry Air | Zero grade (best available) | | < 2 v.p.m. | GC |
| Carbon Dioxide | 99.995% | | < 4 v.p.m. | Ref gas |
| Nitrogen | 99.999% | < 1 v.p.m. | < 1 v.p.m. | Ref gas |
| Nitrous Oxide | 99.9% | < 1 v.p.m. | < 4 v.p.m. | Ref gas |
| Sulphur dioxide | 99.9% | | | Ref gas |



Warning: Compressed gas cylinders should be securely fastened to an immovable structure or permanent wall. Compressed gasses should be stored and handled in accordance with relevant safety codes.



Warning: To avoid possible eye injury, wear eye protection when using compressed gas.

Clean, two stage regulators must be fitted on the gas supplies. These should have stainless steel diaphragms. The second stage regulator must be capable of delivery pressures up to 6 Bar at the instrument.

The cylinder regulators or intermediate supply pipework should be fitted with connectors for 1/8" OD stainless steel capillary.

A supply of 1/8" OD stainless steel capillary should be available for connection of the instrument. Tubing should be of chromatography grade and free from oil or other contaminants. The lengths required will be determined by the location of the cylinders (or intermediate pipework) terminations and the intended placement of the instrument.

Note: For the reference gas pipework it is advisable to locate the cylinders as close to the instrument as possible. This reduces the possible sources of contamination of the reference gas and minimises the required purge time.

Should the cylinders be located some distance from the instrument, consideration should be given to the use of larger diameter tubing to reduce pressure drop across the supply pipe.

Lifting requirements

The components that comprise your IsoPrime instrument may be of considerable weight and may therefore pose a safety hazard when lifting, lowering or moving them.

IsoPrime-EA User Manual

UK health and safety guidelines (Manual Handling Operations Regulations 1992) require that all lifting/lowering operations are carried out by trained personnel and are planned before commencement.

As a general guide, before undertaking any lifting, lowering or moving operation:

- Assess the risk of injury
- Take action to eliminate risk

If some risk still exists:

- Plan the operation in advance and in conjunction with our engineer when he/she arrives on site.
- Use trained personnel where necessary.
- Adhere to appropriate country and/or company regulations.



Warning: Persons with a medical condition, for example a back injury, which prevents them from handling heavy loads should not attempt to lift the heavy components of the instrument. Micromass accepts no responsibility for any injuries or damage sustained while lifting the instrument.

Micromass personnel are not permitted to manually lift the instrument without suitable assistance from local site personnel.

The following information is provided to aid in planning lifting/lowering operations.

IsoPrime cubicle

The IsoPrime cubicle may weigh up to 82kg when fitted with optional equipment.

Suitably trained personnel will be required to raise or lower the cubicle, either manually or with the use of lifting equipment.

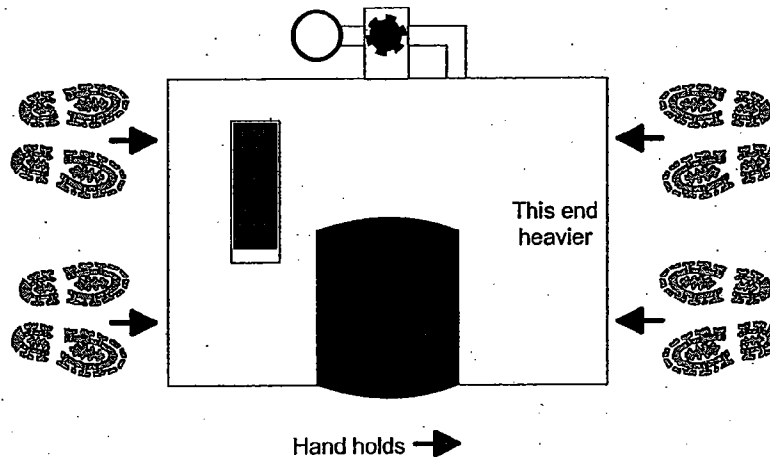
The cubicle lid must be removed before lifting (Open the lid to the vertical position and lift the hinges from their sockets), This brings the total weight to 77kg.

The UK health and safety guidelines recommend that a minimum of four trained and physically suitable personnel are required to lift a unit of this weight from the packing case to the bench. The personnel should be positioned for equal distribution of the load.

All other lifting operations should be assessed before commencement.

Lifting points for manual lifting

The cubicle should be lifted by four people positioned as shown in the diagram below.



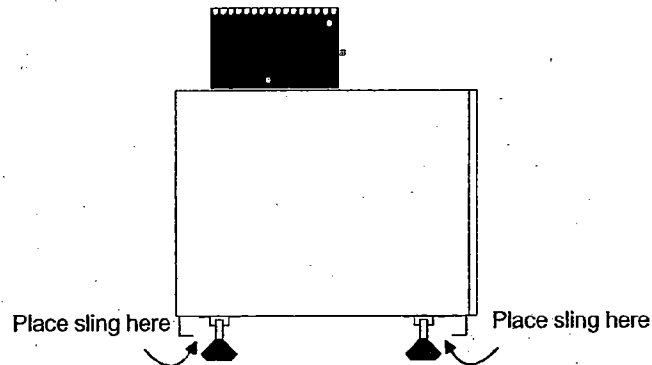
Caution: Under no circumstances should the instrument be lifted by the doors, foreline-connection or lid. Hands should be placed underneath the instrument and support from the bottom.

Lifting points for mechanical lifting

The cubicle lid should be removed before slinging the instrument (Open the lid to the vertical position and lift the hinges from their sockets).

The sling should be run under the lip of the cubicle base-plate and in front of the cubicle feet as shown in the diagram below.

Care should be taken to stop the heavy end of the instrument from twisting in the sling.



Permanent magnet

The permanent magnet weighs 21kg. Lifting the permanent magnet from the packing case to the bench has been assessed as being within the safe lifting capability of two, trained, people.

Lifting the permanent magnet from the bench to the mounting position, has been assessed as being within the safe lifting capability of a single, trained, person.

All other lifting operations should be assessed before commencement.

Electromagnet

The electromagnet weighs 45kg. Suitably trained personnel will be required to raise or lower the magnet, either manually or with the use of lifting equipment.

The UK health and safety guidelines recommend that a minimum of three trained and physically suitable personnel are required to lift a unit of this weight from the packing case to the bench, or from the bench to the mounting position.

Personnel should be positioned for equal distribution of the load.

A lifting cradle is supplied with the instrument for manual or mechanical lifting and must be used for safe handling of the electromagnet.

Computer

The computer system is composed of a number of individual components. Each component is normally within the safe lifting capability of a single trained person.

The manufacturers documentation should be checked for specific handling recommendations before lifting.

Rotary pump

The RV3 rotary pump weighs 23kg.

The manufacturers documentation should be checked for specific handling recommendations before lifting.

GC-Interface

The GC interface is mounted on a wheeled table. Under normal circumstances lifting should not be required. If lifting is required then an assessment of the operation must be performed before proceeding.

Caution: The GC is not fixed to the table. Any lifting operation must secure the GC from movement.



µgas interface

The µgas interface is composed of a number of individual, connected, units. The interface should never be lifted as an assembly as the individual components are not tied together.

The individual components and their weights are given in the table below.

| Component | Weight /kg |
|---|------------|
| Oven housing | 11 |
| Sample tray (including racks and tubes) | 4.5 |
| Gilson autosampler | 15 |
| Gilson support | 2.5 |
| Oven/gas control unit | 10 |

Lifting the individual components from the packing case to the bench, and from the bench to the mounting position, has been assessed as being within the safe lifting capability of a single, trained, person.

All other lifting operations should be assessed before commencement.

EA interface

The elemental analyser weighs 130kg and the manufacturers guidelines for lifting and moving should be followed. See the manufacturers documentation for specific handling recommendations.

The EA diluter is within the safe lifting capability of a single, trained, person.

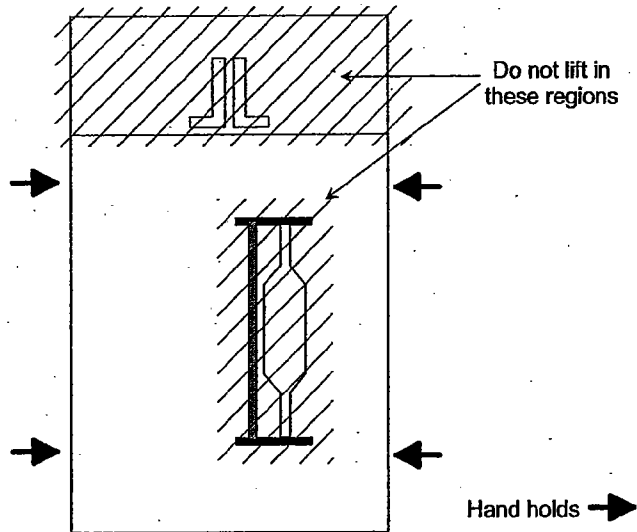
All other lifting operations should be assessed before commencement.

Trace gas preconcentrator

The Trace gas preconcentrator weighs 33kg.

The UK health and safety guidelines recommend that a minimum of two trained and physically suitable personnel are required to lift a unit of this weight from ankle height to the bench. The personnel should be positioned for equal distribution of the load.

The Trace gas preconcentrator should be lifted by two people positioned as shown in the diagram below.



Caution: Under no circumstances should the instrument be lifted by the rear of the instrument where indicated on the diagram or by the sample tube support.

Technical specification

Mass range

1-70 AMU (using electromagnet).

Resolution

The analyser is set to a working resolution of 100 (10% valley definition).

H3+ Contribution (SMOW)

The H3+ contribution is <10 PPM/nA.

Analyser continuous flow specifications

Performed using a reference gas box and continuous flow interface.

Applies to all continuous flow modules.

Reference gas precision ^{13}C

$\leq 0.1\text{‰}$ (SD 1 σ ; on the fit of 10 consecutive pulses of 5nA height).

Reference gas precision ^{15}N

$\leq 0.1\text{‰}$ (SD 1 σ ; on the fit of 10 consecutive pulses of 5nA height).

Reference gas linearity ^{13}C

$\leq 0.3\text{‰}$ (SD 1 σ ; on the fit of 10 pulses varying in height between 1-10nA).

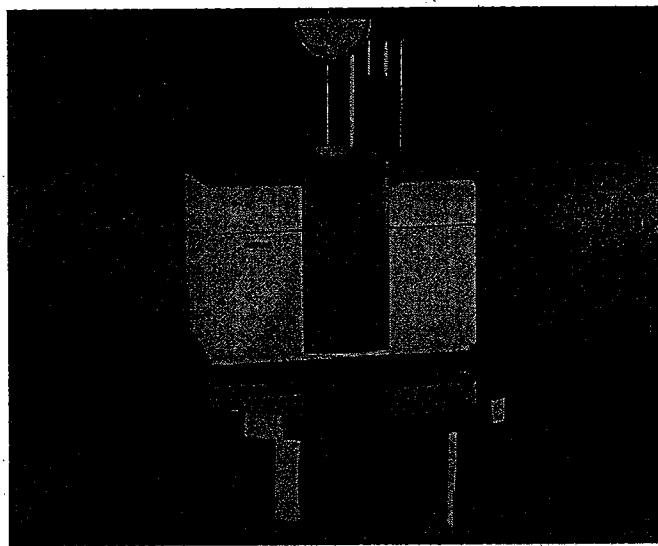
Reference gas linearity ^{15}N

$\leq 0.3\text{‰}$ (SD 1 σ ; on the fit of 10 pulses varying in height between 1-10nA).

IsoPrime-EA User Manual

IsoPrime EA User Manual
Code No 6666588
Issue 1a

Section 3



Isotope Ratio Mass Spectrometers

IsoPrime-EA User Manual

IsoPrime-EA User Manual

| | |
|--|-----------|
| Introduction | 5 |
| Basic mass spectrometry concepts | 6 |
| Mass Selection | 6 |
| Mass Spectrometers | 6 |
| Analytical and Isotope Ratio Analysers..... | 7 |
| Peak Shape | 8 |
| Dispersion | 9 |
| Resolution | 10 |
| Vacuum System | 12 |
| Why Do We Want a Vacuum?..... | 12 |
| How Do We Measure a Vacuum? | 12 |
| Units of Pressure..... | 12 |
| Gas Flow | 13 |
| Viscous Flow | 13 |
| Molecular Flow | 13 |
| Pumps..... | 13 |
| The Rotary Vane Pump | 14 |
| The Turbo Molecular Pump | 15 |
| Pressure Gauges | 16 |
| Total Pressure Transducer..... | 16 |
| The Pirani Gauge..... | 16 |
| The Ionisation Gauge | 16 |
| The Penning Gauge | 18 |
| Sample Inlet Principles | 19 |
| Continuous Flow Operation..... | 19 |
| Calculations and Corrections..... | 19 |
| Enrichment Delta, δ | 19 |
| Isotopic Abundance (Atom%) | 19 |
| Internal Reproducibility | 20 |
| Instrument Corrections | 20 |
| Carbon Dioxide Corrections (Craig) | 21 |
| Nitrogen Corrections..... | 21 |
| Other Analysis Considerations..... | 23 |
| Sequence of data reduction (General case)..... | 23 |
| A Simple Example for Carbon Dioxide..... | 24 |

IsoPrime-EA User Manual

| | |
|--|----|
| Calculations specific to Carbon Dioxide..... | 24 |
| Santrock and Hayes..... | 24 |
| SMOW..... | 25 |
| Atom % Carbon..... | 25 |
| Calculations Specific to Nitrogen..... | 25 |
| Atom % Nitrogen..... | 25 |
| Enriched Nitrogen Analysis..... | 25 |
| Atom Percent Formulae..... | 26 |
| Carbon Dioxide..... | 26 |
| Nitrogen..... | 27 |
| Oxygen..... | 27 |
| Sulphur Compounds..... | 27 |
| Nitrous Oxide (N ₂ O)..... | 28 |

Introduction

This section of the manual is aimed at those users who are new to isotope ratio mass spectrometers (IRMS) or are unfamiliar with some of the basic concepts of these instruments. This section only touches the surface of the material it could cover. However we feel that it is sufficient in depth to enable the new user to move on to use the instrument with confidence and understanding. Most experienced users will probably ignore this section, however the 'Calculations and Corrections' pages contain definitions of formulae for calculations and corrections generally used and thus may be of interest as a reference section.

This is a generalised section and is used in all the user manuals, so please ignore any parts which are not relevant to the system.

Basic mass spectrometry concepts

Mass Selection

If an ion of mass M and charge Z is accelerated in a potential V and injected into a uniform magnetic field B then the ion experiences a force and moves in a circular orbit of radius R . The motion is defined by;

$$\frac{M}{Z} = \frac{B^2 R^2}{2V} \quad (1)$$

for singly-charged ions the radius is determined by the choice of magnetic and electric field. The combination of fields selects ions of particular mass and forms a mass filter. This principle is the basis of all magnetic-sector mass spectrometers and equation (1) is frequently termed the mass spectrometer equation.

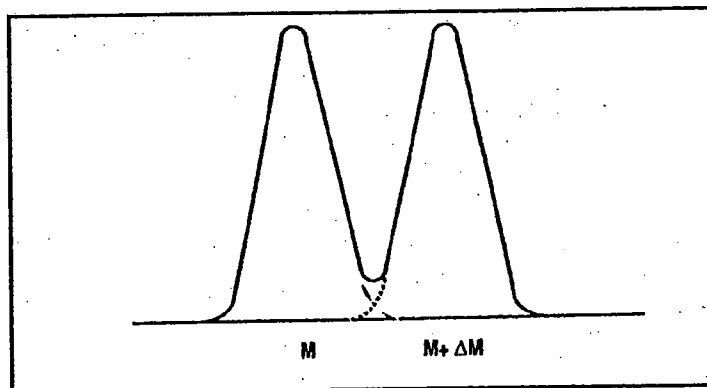
Mass Spectrometers

To analyse a sample gas its molecules must be ionised, formed into a beam, accelerated by an electric field, deflected in a magnetic field, and finally detected. These five processes take place in the analyser of a mass spectrometer, which consists of three separate sections: the source, flight tube, and collector. Ionisation, beam formation and acceleration all occur in the source, magnetic deflection takes place in the flight tube and detection takes place in the collector.

The ionisation is commonly achieved by passing a beam of electrons through the gas sample. Collision or close approach of an electron and sample molecule can cause one or more electrons either to adhere to the molecule and form a negative ion, or to detach from the molecule and leave a positive ion. Except in special cases, it is the singly-charged positive ions (molecules that have lost one electron) which are used in mass analysis. These positive ions are accelerated and formed into a well-defined beam by raising the ionisation chamber to a positive potential and accelerating the ions out through a slit towards a second defining slit at ground potential. The two slits are known as the source and alpha slit respectively.

The flight tube forms an arc of a circle and passes between the poles of a magnet. As the ion beam travels down the tube, it is separated into beams of different radii corresponding to different masses. A particular radius, and hence mass, is selected by a slit at either end of the flight tube; the alpha slit in the source and the resolving slit in the collector.

In the collector, ions of the chosen mass are transmitted through a resolving slit and detected by a Faraday cup. The ion current from the cup is proportional to the number of incident ions and hence to the partial pressure of the corresponding isotopic molecular species in the sample gas. Multiple slits and Faraday cups are frequently used to obtain simultaneous detection of different masses. This method is used in isotope ratio instruments to measure a major beam, due to the most abundant isotopic species of a molecule, and minor beams from the less abundant species.



Mass Separation Diagram

Analytical and Isotope Ratio Analysers

The most pronounced difference between analytical and isotope ratio instruments lies in the peak shape observed by scanning the magnetic or electric fields. In analytical work a mass range is scanned to obtain a spectrum of mass peaks which are characteristic of chemical composition. In isotope ratio work the chemical composition of the sample is known and the fields are held constant so that the variations of isotopes in one chemical species may be measured with high precision. Thus an analytical instrument requires a very narrow peak to distinguish closely spaced masses, whereas a very broad peak is required for high stability in the amplitude measurements of isotope ratio instruments.

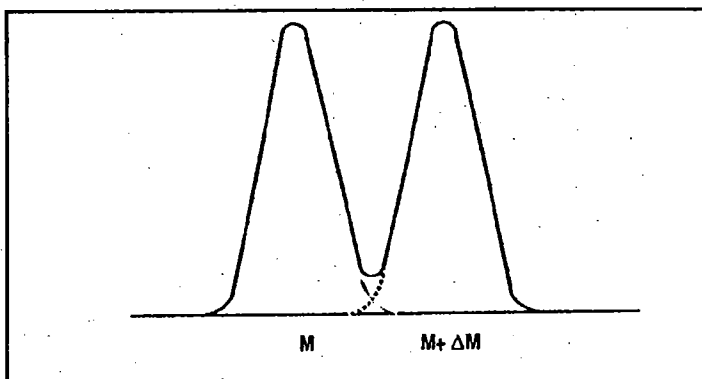
Peak Shape

Peak shape is defined by the source and resolving slits because the analyser is constructed such that the image of the source slit is focused at the resolving slit. (The alpha slit merely eliminates unwanted ions.)

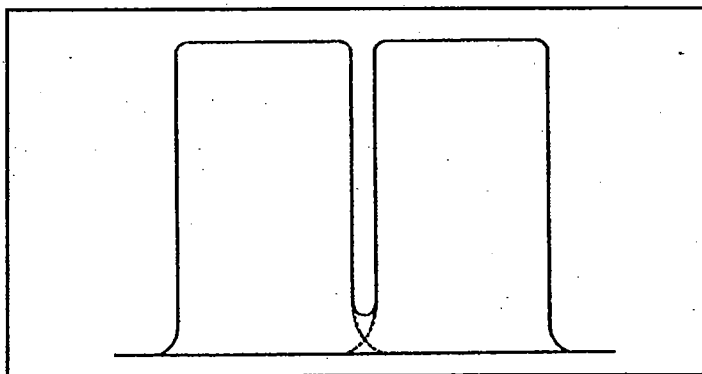
For a resolving slit (at the collector) much narrower than the source slit, a triangular peak shape would be observed as the image of the source slit was swept across the resolving slit (see Narrow Collector Slit figure below). Thus the source slit determines the minimum peak width.

For a resolving slit much wider than the source slit, the image of the source slit would be wholly contained within the resolving slit, and there would be a broad range of electric and magnetic fields over which the image of the same mass could be observed. Although the slopes of the triangular peak would remain, they would be separated by a large mass range over which the signal did not change and a broad flat-topped peak would be observed (see Wide Collector Slit figure below). Thus the resolving slit determines the width of a flat-topped peak, which is limited simply by the encroachment of the adjacent mass peaks.

A narrow source and resolving slit suit analytical measurements, but a narrow source and broad resolving slit best accomplish isotope ratio measurements.



Narrow Collector Slit



Wide Collector Slit

Dispersion

The mass dispersion determines the separation between adjacent mass peaks.

For an analyser of radius r , source slit-to-magnet exit distance x_s and resolving slit-to-magnet exit distance x_r , the mass dispersion measured perpendicular to the ion trajectory is expressed as:-

$$D = \Delta r \left| 1 + \sqrt{\frac{(r^2 + x_r^2)}{(r^2 + x_s^2)}} \right| \quad (2)$$

However, differentiating the mass spectrometer equation ("Mass Selection" above, equation (1) gives:-

$$\frac{\Delta M}{M} = \frac{2\Delta r}{r} \quad (3)$$

and therefore the mass dispersion may be expressed as:-

$$D = \frac{r\Delta M}{2M} \left| 1 + \sqrt{\frac{(r^2 + x_r^2)}{(r^2 + x_s^2)}} \right| \quad (4)$$

In a symmetrical analyser where $x_s = x_r$, the mass dispersion reduces to:-

$$D = 2\Delta r = \frac{r\Delta M}{M} \quad \text{or} \quad D = \frac{r\Delta M}{M} \quad (5)$$

in isotope ratio instruments where peaks are separated by one mass unit. It is clearly seen that dispersion is specified by the radius of a symmetrical instrument and this is frequently quoted as the dispersion itself.

For an asymmetrical analyser, the dispersion is frequently compared to an effective radius

$$r_{\text{eff}} = \frac{r}{2} \left| 1 + \sqrt{\frac{(r^2 + x_r^2)}{(r^2 + x_s^2)}} \right| \quad (6)$$

which combines all the necessary geometric information.

Resolution

Two peaks are completely separated if (neglecting aberrations):

$$D > (W_s + W_r)$$

where W_s is the source slit width
and W_r is the resolving slit width

Mass resolution is defined as

$$R = \frac{M}{\Delta M}$$

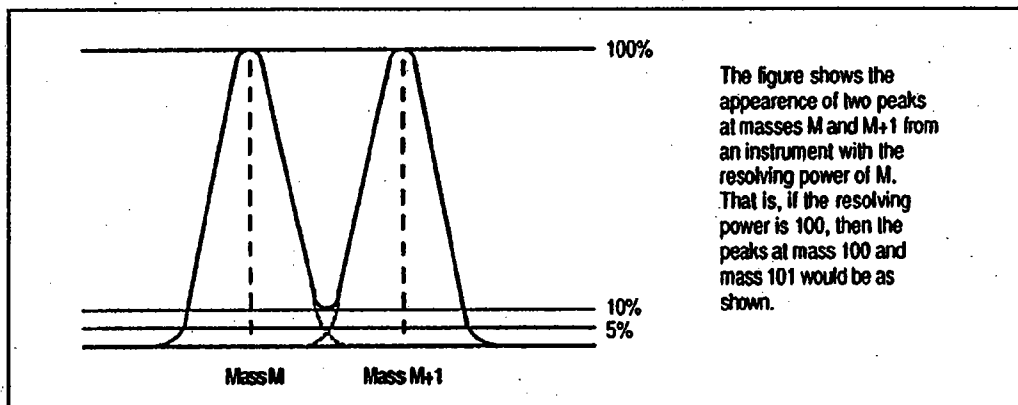
from 5

$$R_{\max} = \frac{r_{\text{eff}}}{D} \quad (7)$$

which (from 6) is a maximum when $D = W_s + W_r$

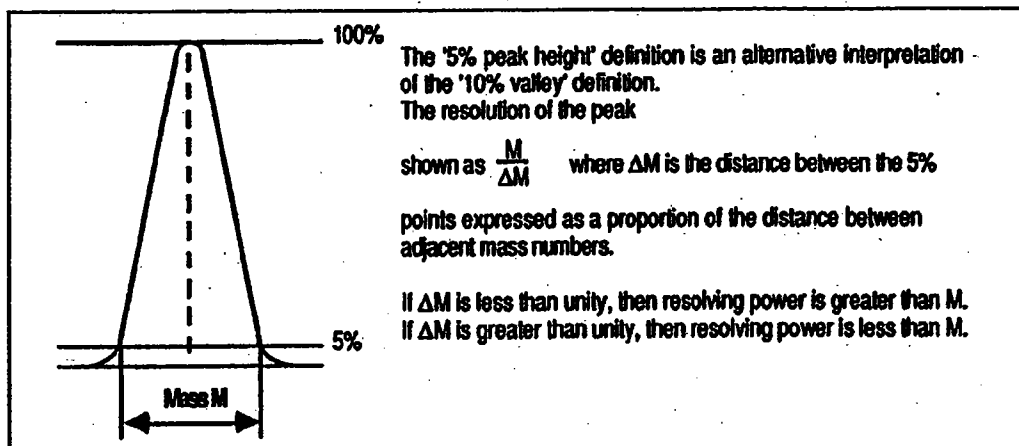
$$\text{i.e. } R = \frac{r_{\text{eff}}}{W_s + W_r} \quad (8)$$

The resolving power of a mass spectrometer is the highest mass number at which peaks of adjacent molecular weight and equal heights have a valley between them of 10% of the peak height. This is known as the 10% valley definition.



Valley Resolution

As an alternative 5% definition is identical to the 10% valley. In this case ΔM is defined as the width of the peak at the 5% height espoused as a proportion of the distance between adjacent masses.



5% Peak Height Resolution

The separation between the peaks is determined by the image size, ions extracted from the source on the wrong trajectory and by aberrations in the optics.

In principle the maximum resolution is directly calculable from the radius and slit width equation (8).

However, the design of the high sensitivity source used in Isotope machines results in a high intensity image which is narrower than the source defining slit and so increases the actual available resolution of the instrument.

Vacuum System

Why Do We Want a Vacuum?

In mass spectrometry we are particularly interested in vacuums for two reasons:-

1. Ion scattering:- If the ions collide with any residual gas molecules their trajectory will be modified resulting in peak broadening.
2. Contamination:- Residual gases in the ionising chamber are also ionised together with the sample material giving rise to an instrument background.

How Do We Measure a Vacuum?

Gases are composed of small particles which are in constant motion. As these particles move around in space they collide with other objects and exert a force. If we can take a unit of area and measure the number and intensity of particle impacts then the resultant is a pressure measurement.

The pressure per unit area P is

$$P = \frac{1}{3} nmv^2$$

where

$$v = \left(\frac{3kT}{m} \right)^{\frac{1}{2}}$$

m is the mass of the molecule,

n the number of molecules in unit volume.

v is the root mean square of all the possible molecular velocities.

k is Boltzmann's constant (1.38×10^{-23}) Joule T^{-1}

and T the temperature of the gas in degrees Kelvin.

Units of Pressure

Atmospheric pressure is equivalent to

| | |
|---------|----------------------|
| 14.7 | lbs per sq inch |
| 760 | mm of Hg |
| 760 | Torr |
| 760000 | millitorr or microns |
| 101325 | Pascal |
| 1.01325 | Bar |
| 1013.25 | millibar |

Common terms for pressure regimes are

| | |
|--------------|---------------------------------|
| Rough vacuum | 10^3 to 10^{-3} millibar |
| High vacuum | 10^{-3} to 10^{-8} millibar |
| UHV | $< 10^{-8}$ millibar |

Gas Flow

The process of evacuation involves the removal of gas from a vacuum vessel. The rate of removal (i.e. the gas flow) determines the rate at which the pressure decreases. It is therefore important to understand viscous and molecular flow regimes.

Viscous Flow

In general gas molecules occupying a space at pressures of greater than 10^{-2} millibar act very much as a fluid. In this viscous flow range the molecules are constantly bumping into each other and are so closely packed together that as the vacuum pump moves some of them out of the chamber others rush to fill that empty space. In viscous flow ranges molecular movement is predictable and smaller diameter hoses and tubes can be used for rough pumping. Viscous flow allows great quantities of molecules to be moved per unit time from one place to another.

Molecular Flow

At lower pressures molecules are so far apart that they no longer exert any influence on each other and motion is strictly random. Depending on pressure a gas molecule may move mm, cm, meters, or even kilometres before it strikes another molecule. This means molecular movement cannot be depended on to push or start a flow pattern. This is why we need to have such large diameters on high vacuum pumping systems, as then we increase the probability that one of these randomly moving molecules will move into the pump.

The difference between the flow regimes does not depend solely on pressure but also on the dimensions of the vacuum container, pipes, etc. It basically depends on the mean free path of the gas molecules.

Pumps

The purpose of a pump is to provide a means for removing gas. Its pumping speed S is rated in litres per second. Most practical pumps operate continuously so that if a constant flow of gas Q is introduced into the pump then a steady state is set up with an associated constant pressure within the pump. The pumping SPEED is defined as

$$S = \frac{Q}{P}$$

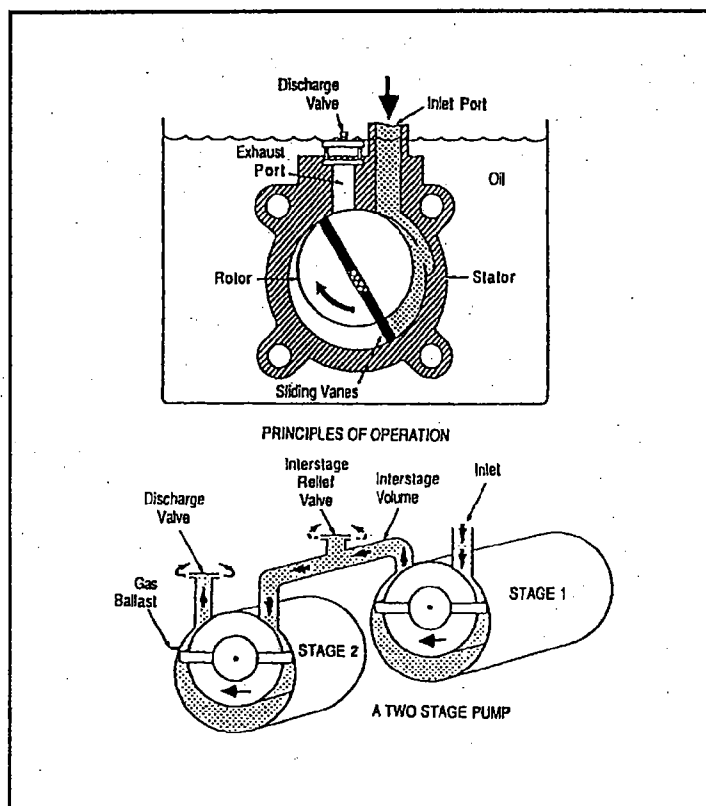
and is clearly an indication of pumping ability.

Air is easily removed at high pressures but after a short while very little gas remains so that pumping speed does not indicate how many molecules are being removing from the system. The amount of gas present in the system is determined by multiplying the pressure (in torr) by the volume (in litres) which tells us the number of molecules in the system or the gas LOAD. Apart from back streaming of pump fluids and influx from real leaks, there are four main sources of gas load in a high vacuum system: (1) desorption of absorbed gases, (2) volatilisation, (3) diffusion of gases from inside solids and (4) gas permeation.

In vacuum systems we are interested to know how much work has to be done to transfer a mass of gas from one place to another.

The Rotary Vane Pump

The rotary vane pump removes gases by compressing them to a point slightly above atmospheric pressure by means of an offset rotor with spring loaded vanes. It then expels the gas to the outside world, approximately 99.999% of the air being removed. It is used to produce rough or foreline pressures. The pump is immersed in an oil bath and the oil is purified to remove high vapour pressure contaminants. It serves to cool and lubricate the pump and provides the seal against atmospheric pressure. The pump motor may be directly coupled or belt driven. Most pumps have two stages to produce a better vacuum. As the film of oil makes the final seal the ultimate pressure achievable is partly determined by the vapour pressure of the oil. If the oil becomes too loaded with water or other impurities the pump's performance will be drastically reduced. Clean oil in the rotary pump is therefore very important. When operating at low pressures the rotary vane pump tends to back stream oil vapour to the roughing line which may even migrate to the vacuum chamber. This can be controlled by means of suitable precautions such as foreline traps in the pumping line.



Rotary Vane Pump

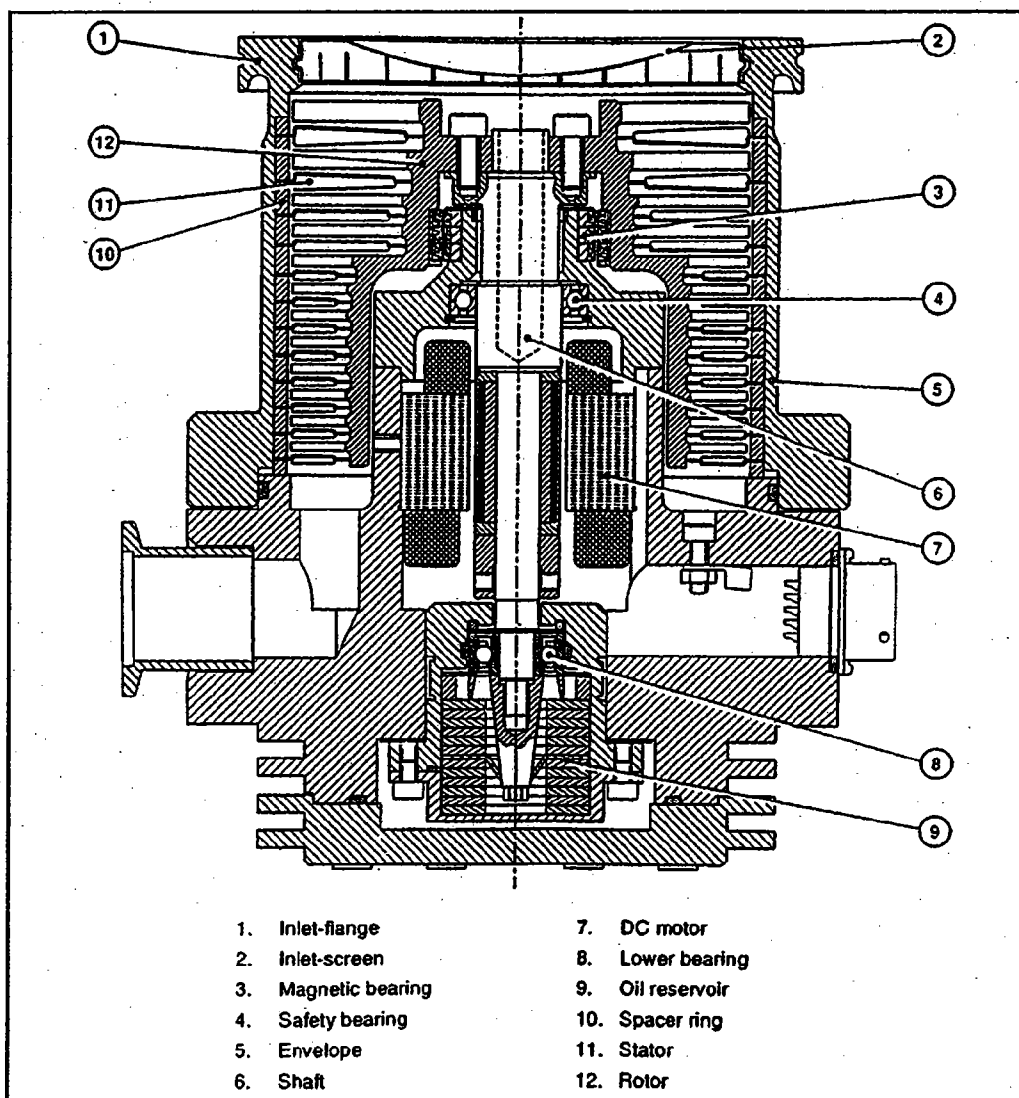
An oil-sealed mechanical pump includes a housing, or stator, an offset rotor with spring loaded vanes, an intake port and an exhaust port equipped with a discharge valve. They may also have a ballast valve. Direct drive pumps usually run at 1725 rpm. For a more detailed description please refer to the pump manufacturers manual.

The Turbo Molecular Pump

A Turbo Molecular pump (TMP) is essentially a high speed axial compressor. Gas is taken from the low vacuum side, compressed and exhausted to the vacuum foreline to be pumped away by the rotary pump.

If the TMP is turned off while open to the vacuum system, hydrocarbons from the foreline and motor bearing will diffuse through the rotor/stator interspaces to the high vacuum part of the pump. A vent valve which admits vent gas to the TMP is opened when the pumping system is turned off and the pump speed has dropped below 50% of the full rotational speed to prevent this back diffusion.

The TMP will only operate if the rotary pump is working. The TMP is self regulating and accelerates as the pressure drops.



Turbo Molecular Pump

For a more detailed description please refer to the pump manufacturers manual.

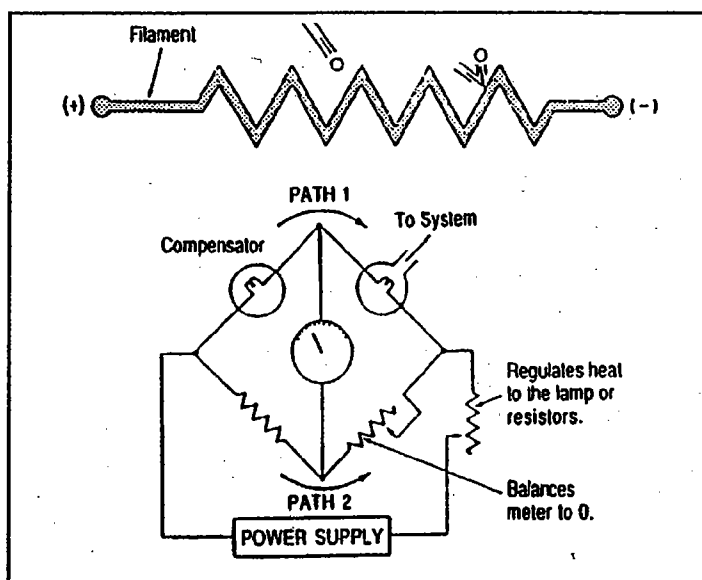
Pressure Gauges

Total Pressure Transducer

This consists of a stainless steel pressure chamber isolated by a stainless steel diaphragm which is resistant to corrosive materials. The inner face of the metal diaphragm has high gain strain gauges bonded to it. When the diaphragm is deflected resulting from a change in pressure in the vacuum system, the strain gauge output changes. This 1-6V output signal is proportional to system pressure and is monitored to give a measure of pressure in mBar.

The Pirani Gauge

This is very similar to the thermocouple gauge in operation in that it relies on gas molecules colliding with a filament conducting heat away. However in this case the filament resistance rather than the temperature is measured. The filament forms part of a bridge circuit. In the balanced bridge circuit the current flow through path one and two is equal and a meter placed at the centre as shown reads zero. When the filament changes resistance the balance is upset and a voltage difference is developed at the meter terminals and a current flows through it. This is calibrated in terms of pressure. The compensator is very similar to the filament and is held at a constant temperature and pressure. The Pirani gauge generally operates in the range 10^3 mBar to 10^{-3} mBar.



Pirani Gauge

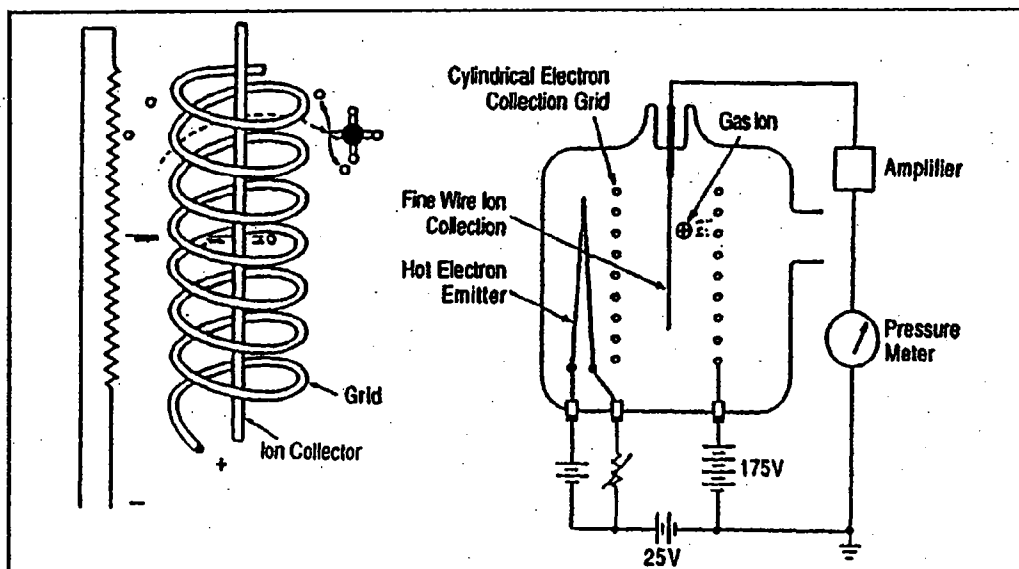
The Ionisation Gauge

The ionisation gauge works on a very similar principle to an ion pump and is unique in that it can operate over 12 orders of magnitude from 10^{-2} mBar to 10^{-14} mBar. It is more commonly used over 6 orders of magnitude from 10^{-4} to 10^{-10} mBar and is expected to be within 30-50% of the correct pressure for high vacuum work.

It consists of a hot filament, a grid, an ion collector and a control unit to provide power, amplification and metering. Electrons from the hot filament are emitted and attracted towards the grid. However many electrons miss the grid wires and swing around it several times before striking it. Because of the large number of electrons emitted from the filament a fairly constant

IsoPrime-EA User Manual

electron cloud is formed about the grid. Many electrons collide with gas molecules ionising them and releasing more electrons. The positively ionised molecules are attracted towards the collector where they produce an ion current proportional to the number of gas molecules in the chamber and hence the pressure. To get meaningful pressure readings the sensitivity of the gauge must be known and the emission current well regulated.



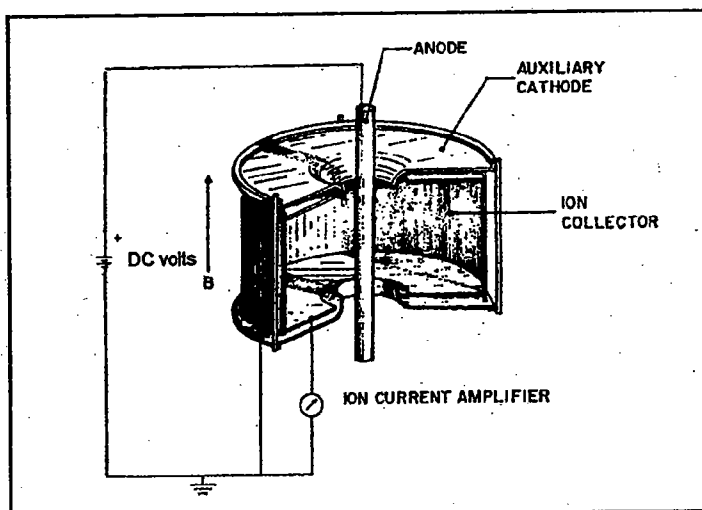
The Ionisation Gauge

It should be noted that sensitivity varies with different gas species. Ion gauges are usually air calibrated and therefore most accurate when analysing nitrogen.

The ionisation gauge like any other has its limitation. Low energy X-rays are created when the electrons strike the grid which causes the releases of photo-electrons and hence a constant error signal. Above 10^{-10} mBar this is insignificant but at lower pressures, corrections must be made for this X-ray effect.

The Penning Gauge

The Penning Gauge operates on the principle that, at pressures below 100mBar, the ionisation current flowing between two electrodes varies with pressure. The interior of the head is open to the vacuum system and contains a rod anode surrounded by a cylindrical cathode. A magnetic field is maintained in the gauge by an external permanent magnet.



Simplified Penning Gauge Diagram

A DC voltage is applied between the anode and cathode which causes free electrons, which are accelerated from the cathode toward the anode. These electrons collide with any gas molecules present causing ionisation. The resulting electrons and positive ions are also accelerated by the electrostatic field causing further ionisation. The mean path length of each ion is extended as they follow a spiral path in the magnetic field. The net current flowing between the anode and cathode is then detected by the control unit and is a function of the pressure in the gauge head.

Notes:

- a) It should be noted that sensitivity varies with different gas species. Penning gauges are usually air calibrated and therefore most accurate when analysing nitrogen.
- b) Prolonged operation of the Penning gauge head above a pressure of 10^{-2} mBar is not recommended as permanent damage can be caused by sputtering.

The time taken for the gauge head to 'strike' (start reading the pressure) increases with decreasing pressure.

Sample Inlet Principles

Continuous Flow Operation

The Continuous Flow Inlet comprises of two vitreous silica capillaries which continuously carry Helium gas into the ion source of the mass spectrometer. These two capillaries draw Helium at two open splits, one fed from a reference gas injector system, the other from a sample preparation system (e.g. gas chromatograph, elemental analyser or μ gas injector).

At user defined times the reference gas injector pulses rectangular peaks of pure reference gas, (pre-calibrated against an international standard) into the ion source. The sample gases exiting from a gas chromatographic column are carried by a stream of Helium towards the sample open split, from here the sample gases also enter the ion source.

The areas under the sample and reference peaks are calculated, from which the difference in isotopic composition between the two is evaluated. This difference is expressed in delta notation, details of which can be found in the Calculation and Corrections section below.

Calculations and Corrections

Enrichment Delta, δ

Enrichment is the excess of any particular isotope species in the sample compared to the same isotopic species in the reference. The enrichment delta expresses this excess as a fraction of the particular isotopic species in the reference and is almost universally quoted in parts per thousand and written as ‰ or per mil. Thus the enrichment delta is;

$$\delta = \left(\frac{R_{sam} - R_{ref}}{R_{ref}} \times 1000 \right) \text{‰}$$

Where R_{sam} and R_{ref} are the sample and reference ratios of minor to major beam as measured on the isotope ratio instrument.

Enrichment delta notation can be used to express results for all species of gases measured.

Isotopic Abundance (Atom%)

This expresses the number of atoms of a particular isotope of an element in the sample as a fraction of the total number of atoms of that element present. It is usually expressed as a percentage and notated as Atom% or At%.

This notation is particularly popular amongst the biological users and for nitrogen measurement can be defined as:-

$$\text{Atom\%} = \frac{\text{number_of_}^{15}\text{N_atoms}}{(\text{number_of_}^{15}\text{N_atoms}) + (\text{number_of_}^{14}\text{N_atoms})}$$

Some users prefer to express their results as atom% excess (i.e. atom% enrichment in excess of the reference gas) which is defined as

Atom% excess = sample atom% - reference atom%

Internal Reproducibility

In a set of n measurements of a quantity x whose mean is x_{mean} there are two commonly used and related quantities for the precision.

Standard deviations of a measurement, σ

This is also referred to as the standard deviation and defines the precision of one individual measurement from the complete set of measurements. It is estimated from the data by

$$\sigma = \sqrt{\frac{\sum (x - x_{mean})^2}{(n-1)}}$$

Standard deviation of the mean, σ_{mean}

This is also referred to as the standard error and defines the precision of the average over the complete set of measurements. It is rigorously related to the standard deviation by

$$\sigma_{mean} = \frac{\sigma}{\sqrt{n}} = \sqrt{\frac{\sum (x - x_{mean})^2}{n(n-1)}}$$

The internal reproducibility of an instrument is defined as its standard error, where n is the number of values of delta produced by one set of sample/reference comparisons. For example a set of 6 pairs of sample/reference comparisons producing 10 values of delta would give

$$\sigma_{mean} = \sqrt{\frac{\sum (x - x_{mean})^2}{90}}$$

Instrument Corrections

In isotope ratio instruments there are three correction factors which may be of concern

A tail contribution from major to minor isotope peak.(abundance sensitivity correction)

A background gas peak at the minor isotope mass.

If the vacuum system is clean, background corrections also should be ignored, although software facilities are available to subtract background readings from all acquired data if required. If the sample has been prepared incorrectly, there may be problems due to contaminants at the masses of interest (e.g. CO peaks overlapping with nitrogen peaks in a dirty nitrogen sample). This type of interference may cause small errors in delta values. The peak jump facilities in the software are provided in order to assess the nature of possible contaminants.

Carbon Dioxide Corrections (Craig)

When analysing CO_2 different isotopic species of the same element can produce a contribution at certain masses and a correction must be made. For example the international CO_2 standard PDB has a contribution (approximately 6%) at mass 45 due to the ^{17}O isotope. Similarly, 0.2% of mass 46 is derived from isotopic species containing ^{13}C and ^{17}O but not ^{18}O . Masses higher than 46 have negligible abundance's.

For a triple collector instrument measuring a sample close to PDB, the correction formulae are

$$\delta^{13}C = 1.0676\delta(45/44) - 0.0338\delta^{18}O$$

and

$$\delta^{18}O = 1.0010\delta(46/44) - 0.0021\delta^{13}C$$

Nitrogen Corrections

When analysing nitrogen a problem can, at low enrichments, arise owing to trace nitrous oxide (NO) causing an interfering peak at mass 30. This, if not correctly taken into consideration, can adversely affect the resultant calculation of ^{15}N atom%.

To overcome this problem it is common to use a formula modified from the standard formulae when the enrichments are low (less than 5%). The two formulae commonly used are:

Standard formula for high enrichments

$$^{15}N_{atom\%} = \frac{R_{29} + 2R_{30}}{2(1 + R_{29} + R_{30})} \times 100 \quad (1)$$

modified formula for low enrichment

$$^{15}N_{atom\%} = \frac{R_{29}}{2 + R_{29}} \times 100 \quad (2)$$

where R_{29} = 29/28 mass ratio

and R_{30} = 30/28 mass ratio

Derivation of formula used

Given the probabilities of $^{15}N = \alpha$ and $^{14}N = 1 - \alpha$, then assuming the nitrogen sample is in equilibrium the N_2 probabilities can be expressed as:-

$$mass_{28}(\text{denoted } I_{28}) = (1 - \alpha)^2$$

$$mass_{29}(\text{denoted } I_{29}) = 2\alpha(1 - \alpha)$$

$$mass_{30}(\text{denoted } I_{30}) = \alpha^2$$

IsoPrime-EA User Manual

From The following 29/28 and 30/28 ratios can be expressed as:

$$R_{30} = \frac{\alpha^2}{(1-\alpha)^2} \quad \text{and} \quad R_{29} = \frac{2\alpha}{1-\alpha}$$

hence giving $R_{30} = \left(\frac{R_{29}}{2}\right)^2$ (3)

Atom% can be written as

$$\frac{2(^{15}\text{N}^{15}\text{N}) + (^{15}\text{N}^{14}\text{N})}{2(^{14}\text{N}^{14}\text{N}) + 2(^{14}\text{N}^{15}\text{N}) + 2(^{15}\text{N}^{15}\text{N})} \quad (4)$$

$$\text{Atom}\% = \frac{2I_{30} + I_{29}}{2I_{28} + 2I_{29} + 2I_{30}} \times 100 \quad (5)$$

Giving the standard high enrichment formulae (1)

$$\text{Atom}\% = \frac{R_{29} + 2R_{30}}{2(1 + R_{29} + R_{30})} \times 100 \quad (1)$$

Substituting (3) in (1)

$$\text{Atom}\% = \frac{2\left(\frac{R_{29}}{2}\right)^2 + R_{29}}{2\left(1 + R_{29} + \left(\frac{R_{29}}{2}\right)^2\right)} \times 100 \quad (6)$$

$$\text{Atom}\% = \frac{R_{29}^2 + 2R_{29}}{4\left(1 + R_{29} + \frac{R_{29}^2}{4}\right)} \times 100 \quad (7)$$

$$\text{Atom}\% = \frac{R_{29}(R_{29} + 2)}{(R_{29} + 2)(R_{29} + 2)} \times 100 \quad (8)$$

which gives the modified low enrichment formulae

$$\text{Atom}\% = \frac{R_{29}}{2 + R_{29}} \times 100 \quad (9)$$

Both formulae (1) and (2) have limitations:-

formula (1) although accurate at high enrichments can cause errors at low enrichments due to the presence of Nitrous Oxide.

formula (2) overcomes the NO constitution problem at low enrichments by substitution of R_{29} ratios, however it relies on equilibrium of sample nitrogen, thus causing possible errors at high enrichments.

The normal method is to switch between the two formulae at a particular enrichment. The level of enrichment that is decided is dependant on the sample nitrogen. i.e. how well it is equilibrated and its NO concentration.

Other Analysis Considerations

Note: The following sections are provided to allow users to fully understand the data reduction algorithms used in the program. Users may wish to skip this section on first reading.

Sequence of data reduction (General case)

The following calculations are performed where necessary for any gas:

Convert the reference gas deltas to the standard format for further calculation.

For example if CO₂ is being analysed and the reference deltas are input as delta(13) and delta(18), it is necessary to convert these values to delta(45) and delta(46) for further use. This is done automatically if required using the Craig formulae.

Convert the sample delta, measured with respect to the laboratory standard, to the delta w.r.t. the international standard.

This is done using the following formula:

$$\delta(\text{sam w.r.t. international}) = \delta(\text{sample}) + \delta(\text{ref w.r.t. international}) + \frac{\delta(\text{sample}) * \delta(\text{ref w.r.t. international})}{1000}$$

Convert the observed delta values of a gas comprising more than one element to the delta values for the pure element.

The most familiar example is probably the Craig correction for CO₂ mentioned above. The other common case is for sulphur analyses using the 64 and 66 peaks of SO₂. To allow these calculations to be undertaken in the general case a matrix is set up using constants from the parameter file (only accessible at supervisor level) such that:

$$\delta 1(\text{corrected}) = C1 * \delta 1 - C2 * \delta 2(\text{corrected})$$

$$\text{and } \delta 2(\text{corrected}) = C3 * \delta 2 - C4 * \delta 1(\text{corrected})$$

This format has been chosen to closely represent the original Craig formulae. A little thought shows :

| | CO ₂ | CO | SO ₂ | N ₂ | HD |
|----|-----------------|--------|-----------------|----------------|----|
| C1 | 1.0676 | 1.0378 | 1.09 | 1 | 1 |
| C2 | 0.0338 | 0.0169 | 0 | 0 | 0 |
| C3 | 1.0010 | 1.0010 | 0 | 1 | 0 |
| C4 | 0.0021 | 0.0021 | 0 | 0 | 0 |

This general approach allows most gas species to be analysed by determining the values of the four constants. It also allows for non standard collector arrangements to be utilised by careful choice of constants.

A number of preparation systems rely on equilibrium states being reached, rather than reactions proceeding to completion. Examples include the carbonate reaction with acid, the carbon dioxide equilibration over water for oxygen analysis and the hydrogen equilibration over water for hydrogen analysis. The equilibration can be treated in a general way as

$$\delta(\text{true}) = \frac{(\delta(\text{measured}) - 1000 * (E - 1))}{T(\text{standard})} + K * (T(\text{reaction}) - E)$$

IsoPrime-EA User Manual

By specifying E as 1 and K as 0 this formula can be applied to any delta as it will not change the result. **THIS IS THE DEFAULT AND SHOULD NOT BE CHANGED UNLESS A CORRECTION IS REQUIRED.**

For the determination of the oxygen isotope ratio in waters by equilibrium the constants are :

$$E = 1.04115 \text{ and } K = 0.17 \text{ (approx)}$$

whilst in the carbonate reaction using phosphoric acid:

$$E = 1.01025 \text{ and } K = 0.04.$$

The value of T(standard) is normally 25°C. These constants are input in the gas parameter files.

A Simple Example for Carbon Dioxide

If the delta 13 and delta 18 of the reference gas are input these are converted to delta 45 and delta 46 using:

$$\delta 45 = (\delta 13 + (C2 * \delta 18)) / C1$$

$$\delta 46 = (\delta 18 + (C4 * \delta 13)) / C3$$

The sample deltas measured with respect to the lab gas are then modified to be w.r.t. the international standard using the formula given in (2) above.

We can then use the Craig constants again to calculate the delta 13 and 18 of the sample gas relative to the international standard by:

$$\delta 13 = ((C1 * \delta 45) - (C2 * C3 * \delta 46)) / (1 - C2 * C4)$$

$$\text{and } \delta 18 = ((C3 * \delta 46) - (C1 * C4 * \delta 45)) / (1 - C2 * C4)$$

If the corrections are done in any other order the Craig constants (which apply for PDB only) would have to be re-calculated, a point often missed.

Calculations specific to Carbon Dioxide

Santrock and Hayes

These authors have suggested an alternative algorithm for converting delta 45 and 46 to delta 13 and 18 which has yet (at the time of writing) not achieved international recognition. They start from the general formula relating the oxygen 17 to oxygen 18 ratios of:

$$^{17}\text{R} = K(^{18}\text{R})^\alpha$$

The derivation of Craig which is used by most users assumes that the constant (α) has the value of 0.5 and permits an exact derivation of the corresponding equations to determine delta 13 and 18. In the general case considered by the above authors an iteration approach is required. The values of the constants K and α are not well determined. At the time of writing the "best" values appear to be:

$$\alpha = 0.516 \text{ and}$$

$$K = 0.0099235.$$

Two other constants are required for this analysis, namely the carbon 13 to carbon 12 ratio and the oxygen 18 to oxygen 16 ratio in the international standard PDB. (The oxygen 17 ratios are obviously determined in the calculation using the above formula). These values are:

$$^{13}\text{R}_{\text{PDB}} = 0.0112372$$

$$^{18}\text{R}_{\text{PDB}} = 0.002079$$

IsoPrime-EA User Manual

If the Santrock method is required the user is responsible for determining that the constants employed are correct, otherwise the final reported data will be in error.

These constants can be inserted in the parameter file, accessible only at supervisor level. For further discussion of this technique the reader is referred to the original papers:

Santrock, Studley and Hayes: Anal. Chem 57 1444-8 (1985) and

Bakke, Beaty and Hayes - Paper presented at GSA 1991.

Finally it should be noted that (obviously for consistency) if this approach is to be used the reference gas deltas should be converted to a delta 45 and delta 46 value by the same technique off-line, and these values loaded into the appropriate parameter file. If the delta 13 and 18 values are input the Craig method is automatically employed to do this calculation.

SMOW

Since two international standards for oxygen isotopes exist, PDB and SMOW, it is necessary to be able to report the analyses relative to both standards. The formula used is:

$$\delta 18(\text{SMOW}) = \delta 18 * E + 1000 * (E-1)$$

Where the equilibrium constant E has the value 1.03086.

Atom % Carbon

Once the enrichment of the sample has been determined relative to a reference, it is straightforward to calculate the carbon atom % composition of this sample, using the known composition of the reference. The three constants used in this calculation (13R, 18R and 17R) specified in the parameter file supplied (accessible at supervisor level only) refer to the international standard PDB. If an alternative standard is required it is left to the user to determine the required constant values.

Calculations Specific to Nitrogen

Atom % Nitrogen

Once the enrichment of the sample has been determined relative to a reference, it is straightforward to calculate the nitrogen atom % composition of this sample, using the known composition of the reference. The single constant used in this calculation (¹⁵R) specified in the parameter file supplied (accessible at supervisor level only) refers to the international standard of the nitrogen in the air. If an alternative standard is required it is left to the user to determine the required constant value.

Enriched Nitrogen Analysis

It is sometimes necessary to determine the nitrogen isotopic composition of a sample which is close to natural but where the ^{15,15}N₂ is not in thermodynamic equilibrium with the ^{14,14}N₂ and ^{15,14}N₂. In this case the simple formulae given above may not be used and the analysis must take account of the 28, 29 and mass 30 signals. A problem is often encountered with trying to use the mass 30 signal in that the recorded signal is due not only to a contribution of nitrogen (which is the signal of interest), but also a large contribution from NO is often seen, especially if even a minute trace of air is present in the samples being analysed.

To permit this analysis to be undertaken the following approach is used:

IsoPrime-EA User Manual

- The delta 29 and delta 30 of the sample is calculated in the normal manner.
- From the calculated atom % of the reference gas (obtained from the input atom percent of the international standard and the reference gas delta value) the expected 30 AMU signal is calculated and the difference between this value and the observed is assumed to be the unwanted contribution from NO. This calculation assumes that the mass 30 of the reference gas is in thermodynamic equilibrium (otherwise no method exists to determine the NO signal in the general case).
- The assumption is also made that this contribution is also present in the sample gas (i.e. that they have both similar oxygen impurities or that the major source of oxygen is elsewhere, e.g. from the filament) and the mass 30 AMU signal modified accordingly. The correction used is weighed so that at natural abundance the full correction is used whilst at 100% enrichment the correction falls to zero.
- This corrected 30 signal is then the value used in the subsequent atom % calculation.

If it is felt this approach is inappropriate for the samples being analysed it is left to the user to convert the measured deltas to atom % off line. The above method has proved quite successful at a number of sites where labelling with $^{15,15}\text{N}_2$ is performed. Obviously these difficult experiments in the general case may render the above approach inappropriate, but the user should be aware of the problems which may result from this NO contamination.

Atom Percent Formulae

We now list, for reference, the atom percent formulae use in the code. Note that these equations assume equilibrium thermodynamics in all cases except for enriched Nitrogen analyses where the 30 AMU intensity is monitored. Even here the Reference gas must be in equilibrium, and this specific case is dealt with above.

Carbon Dioxide

$$\text{APC}(^{13}\text{C}) = \frac{100 \text{ R13}}{1 + \text{R13}}$$

$$\text{R13} = (\delta 1 / 1000 + 1) \text{ R1}$$

$$\text{R18} = (\delta 2 / 1000 + 1) \text{ R3}$$

$$\text{R17} = (0.5 \delta 2 / 1000 + 1) \text{ R2}$$

$$\text{and } \text{APC}(^{18}\text{O}) = \frac{100 \text{ R18}}{1 + \text{R17} + \text{R18}}$$

R1, R2 and R3 are the three reference ratios input into the constants page and refer to 13/12, 17/16 and 18/16 ratios of the international standard.

The same formulae apply to carbon monoxide since we have deconvoluted the δ^{29} and δ^{30} to δ^{13} and δ^{18} .

Nitrogen

The calculation method employed for enriched nitrogen (and samples where the 30AMU signal must be considered) is given above. Here we only consider the case where the 29AMU beam is used.

$$\text{APC}(^{15}\text{N}) = \frac{100 \text{ R}_{29}}{2 + \text{R}_{29}}$$

Thus for hypothetical gas consisting of the international standard:

$$\text{R}_{29\text{calc}}^{\text{ref}} = \frac{2 \text{ APC}^{\text{input}}}{100 - \text{APC}^{\text{input}}}$$

From the observed sample delta (with respect to the international standard used, normally air), a calculated sample ratio $\text{R}_{29\text{calc}}^{\text{sam}}$ is obtained, from which the sample atom percent is calculated.

The APC of the international reference is input into the R1 field in the constants page.

Oxygen

We assume that beams 32,33 and 34AMU are measured and that the data is deconvoluted such that $\delta 1$ refers to delta 17 and $\delta 2$ refers to delta 18. Obviously the deconvolution constants must be calculated to refer to the standard gas being used. The definition of the deconvolution constants used in the program is given above.

$$\begin{aligned} \text{R}_{17\text{sample}} &= (\delta 1 / 1000 + 1) \text{R}_{17\text{ref}} \\ \text{R}_{18\text{sample}} &= (\delta 2 / 1000 + 1) \text{R}_{18\text{ref}} \end{aligned}$$

Therefore:

$$\begin{aligned} \text{APC}(^{17}\text{O}) &= \frac{100 * \text{R}_{17}}{1 + \text{R}_{17} + \text{R}_{18}} \\ \text{APC}(^{18}\text{O}) &= \frac{100 * \text{R}_{18}}{1 + \text{R}_{17} + \text{R}_{18}} \end{aligned}$$

The value of $\text{R}_{17\text{ref}}$ is input into the R1 field and the value of $\text{R}_{18\text{ref}}$ into the R2 field of the constants page.

Sulphur Compounds

Since not all the sulphur peaks (due to S32, S33, S34 and S36) are monitored and there does not appear to be a recommended relationship to correlate the variations of one ratio with another, a general formula for the atom percents cannot be given.

Nitrous Oxide (N₂O)

Here again we assume the observed R45 and R46 ratios have been deconvoluted by the user, calculating the constants for the laboratory standard employed and using the general deconvolution parameters as defined above. We assume further that the data is deconvoluted such that $\delta 1$ refers to $\delta 15$ and $\delta 2$ refers to $\delta 18$.

Then:

$$R15_{\text{sample}} = (\delta 1 / 1000 + 1) * R15_{\text{ref}}$$

Where:

$$R15 = \frac{APC15}{APC15}$$

With the APC15 of the reference gas input into the R1 field of the constants page the value of $R15_{\text{sample}}$ is calculated, and the APC of the sample evaluated using:

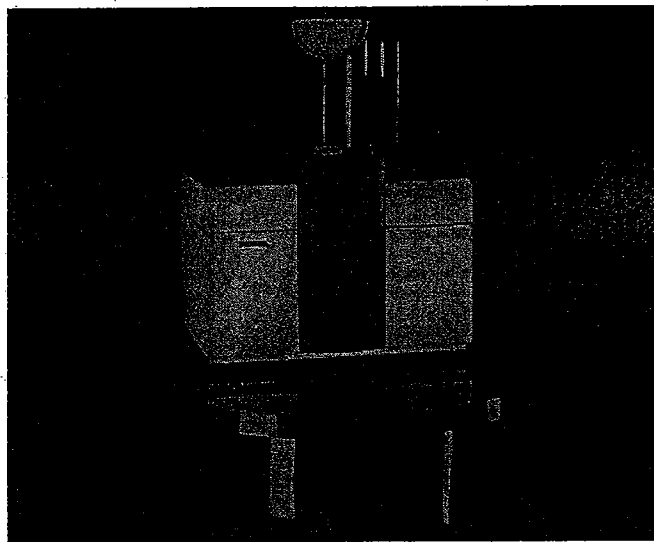
$$APC = \frac{100 * R15}{1 + R15}$$

The Oxygen APC is calculated using the same method as with Carbon Dioxide above.

NOTE: The different definitions for nitrogen and oxygen in these fields.

IsoPrime EA User Manual
Code No 6666588
Issue 1a

Section 4



Equipment Description

IsoPrime-EA User Manual

| | |
|---|-----------|
| Introduction | 5 |
| Mass Spectrometer | 6 |
| Ion Optics Overview | 6 |
| Ion Source | 6 |
| Gas Inlet Probe | 8 |
| Analyser magnet | 9 |
| Permanent magnet | 9 |
| Electromagnet | 10 |
| Analyser housing | 11 |
| Collectors | 11 |
| Vacuum system | 14 |
| Overview | 14 |
| Analyser pumping system | 14 |
| Pressure Measurement | 15 |
| Penning Gauges | 15 |
| Pirani gauges | 16 |
| Vacuum protection | 16 |
| Electrical and Electronics | 17 |
| Overview | 17 |
| Unit Locations | 17 |
| System Controller | 18 |
| Source and High Voltage Supplies | 19 |
| High Voltage Supplies | 19 |
| Source Control Unit | 19 |
| Source connections | 19 |
| Head Amplifier | 19 |
| Electromagnet supply | 20 |
| Turbomolecular Pump Controller | 20 |
| Mains distribution | 21 |

IsoPrime-EA User Manual

| | |
|--|-----------|
| The EA interface | 22 |
| Overview | 22 |
| The Elemental Analyser | 22 |
| Reference Gas Injector | 23 |
| Single gas injector unit | 23 |
| Reference Gas Injection Valve | 23 |
| The Gas Network | 24 |
| The Compressed Air System | 24 |
| Dual gas injector unit | 25 |
| Sample/ reference line interface | 25 |
| The Gas Distribution | 26 |
| Elemental Analyser | 26 |
| Reference gas injection | 26 |
| Utility gas requirements | 26 |
| Operational Overview | 26 |
| CE Instruments NA2500 Elemental Analyser | 27 |
| Interface | 27 |
| Reference gas injection | 27 |
| The Autosampler Carousel | 27 |
| TCD Operation | 27 |
| Data System | 28 |

Introduction

The Micromass IsoPrime has been designed as the base component for a modular isotopic analysis system. Its construction is arranged so that all options and accessories can be added to the basic instrument. This provides the important benefit of a simple upgrade path, so that the system can grow to meet the changing requirements of the user. This section describes the core design of the Micromass IsoPrime, including all the components of the basic instrument.

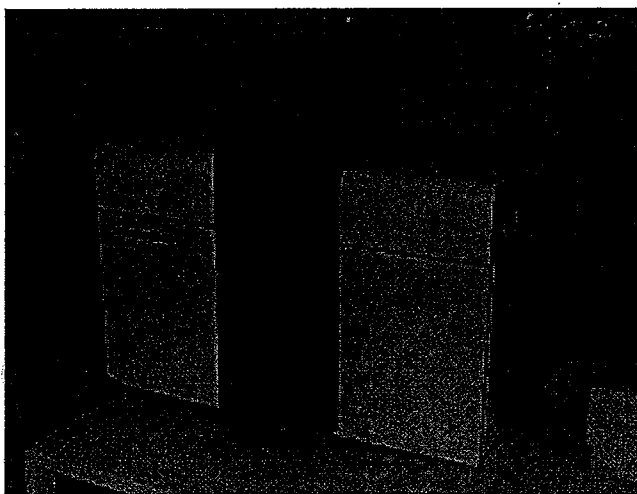
The Micromass IsoPrime is a bench top instrument with a space efficient design. All the major components of the analyser reside within a single cubicle on the bench top. A rotary pump providing roughing vacuum for the IsoPrime is located below the bench.

The result of this arrangement is good ergonomics for a pleasant laboratory environment, with all the main components located for ease of access and maintenance.

The IsoPrime cubicle contains the following components:

- Ion Optics (Mass Spectrometer).
- Inlet isolation valve
- Electronics unit.
- Head amplifier.
- High vacuum pumping system.
- Electrical wiring assembly.

Sample preparation systems are mounted on either side of the IsoPrime cubicle (see the instructions for each interface for specific location requirements).



The Micromass IsoPrime system

Mass Spectrometer

Ion Optics Overview

The ion optics are mounted on top of the IsoPrime case and are contained within a rectangular section vacuum enclosure. The turbomolecular pump extends below the housing within the case. This arrangement provides ready access to the source, collector and isolation valve of the IsoPrime whilst placing the turbomolecular pump within a forced airflow environment. This ensures that the turbomolecular pump is maintained at the correct operating temperature for long and trouble free operation.

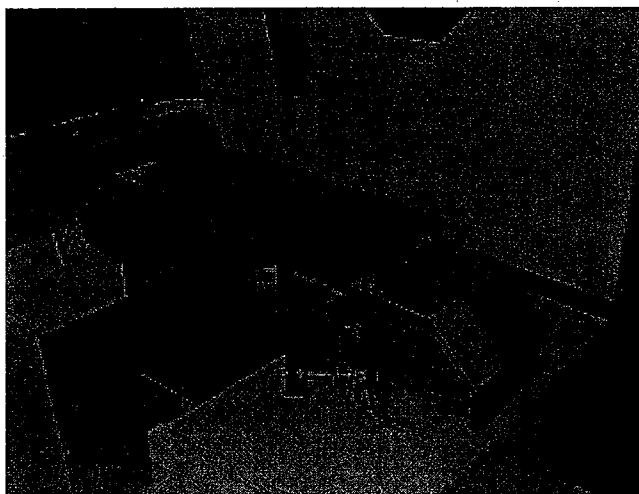
The instrument case is formed from folded steel to provide a rigid and stable base for the ion optics.

The ion optics are arranged to have a horizontal geometry. The source is mounted to the right of the instrument (viewed from the front) with the collectors mounted at the left-hand end of the vacuum enclosure.

The design allows easy access to the source and collectors, which may be removed as complete assemblies on their mounting flanges for ease of maintenance.

The turbomolecular pump is mounted directly under the source to give unrestricted high vacuum pumping. This arrangement produces good abundance sensitivity, low backgrounds, and minimum inter-sample memory.

The IsoPrime is supplied with a hood, hinged at the rear of the cubicle that provides protection for the mass spectrometer or may be lifted to allow access to the vacuum enclosure and magnet.



Ion Optics Housing

Ion Source

The ion source uses the tried and trusted ion optics design of the Optima and Prism instruments. It has been redesigned to make it easier to maintain and assemble.

- The part count has been reduced by 20%.
- Filament replacement has been simplified.

IsoPrime-EA User Manual

- The defining slit may now be replaced without disassembly of the source stack.
- Stack ceramics are now shielded from ion burn for increased lifetime.
- Source wraparound and filament shield design simplified.
- Source connections now push-fit.
- Inlet probe now self-locating for better gas tight fit.



Ion source mounted on the source flange

The ion source is a small chamber in which gas molecules are ionised by collisions with electrons. A source using this form of ionisation is referred to as an Electron Impact (EI) ion source.

The electrons are emitted by thermal excitation from an incandescent wire filament, and are accelerated through the source by a small voltage between the filament and source (electron volts) of 50 -100 volts. The electrons are collimated by a narrow electron entrance slit opposite the filament, and follow a helical path through the source under the influence of the magnetic field produced by two small permanent magnets (the source magnets). This raises the ionisation efficiency by increasing the probability of collisions between gas molecules and electrons.

The majority of electrons, which do not cause ionisation, are collected at the Trap (to produce the Trap current). The current through the trap is used in a feedback loop to control the emission from the filament via the filament current. This is known as a trap-regulated system, and provides a constant flow of electrons through the source.

The source block is a machined component that forms the base and two sides of the ion source block. Machined into the side of the source block is the gas inlet aperture, where the ceramic inlet probe admits gas to the source. Adding the wraparound to the source block forms the source 'box'.

The "Wrap-around" is a photo-etched sheet that forms the top, front and bottom surfaces of the ion box. It contains the electron entrance hole (filament side of the source, referred to as top) and exit hole (trap side, referred to as bottom) on opposite faces, and the ion exit slit on its front face.

IsoPrime-EA User Manual

The ion repeller is a flat electrode at the back of the source box, opposite the ion exit slit, which is used to move the region of ionisation with respect to the slit.

The half plates are two electrodes that form an electrostatic lens, which provides a focusing effect on the ions as they emerge from the ion exit slit. The half plates also provide steering of the ion beam in the y-direction.

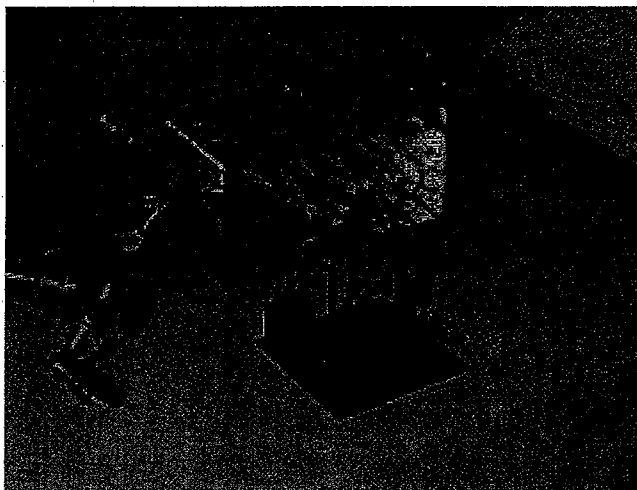
The defining (source) slit ensures that the ion beam is well defined as it leaves the source and is held at ground potential to collect any scattered ions.

The Z plates are two electrodes that provide beam steering in the z-plane.

The final alpha plate defines the maximum beam width at that point prior to the ion beam entering the flight tube on its way to the collectors.

The source voltages and currents are variable and depend on the gas species to be analysed. These voltages and currents are supplied from the electronics units and controlled via the computer (please see later sections of this manual for details of tuning the source and electronic units).

The connections to the source are made via a number of feedthrough connectors located in the source mounting flange.



Source Feedthrough Connections

Gas Inlet Probe

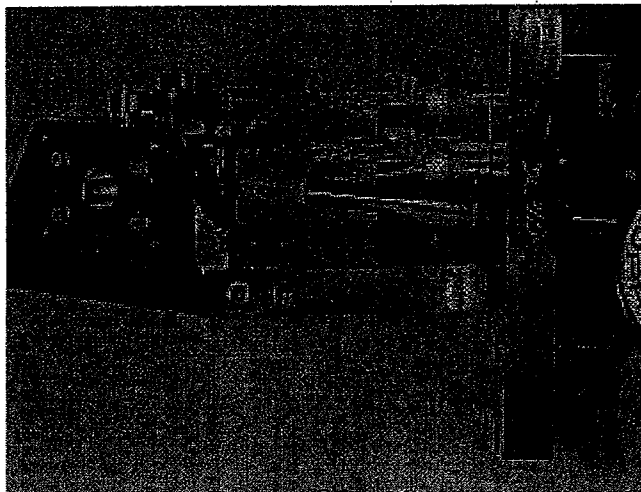
The gas inlet probe of the IsoPrime is composed of three components and is supported by the inlet probe feedthrough that is welded into the source flange.

The stainless steel inlet tube holder slides within the inlet probe feedthrough and is forced towards the source by a compression spring. The end of the inlet tube holder is tapered internally to accept one end of the inlet tube.

The inlet tube is manufactured from ceramic and is tapered on both ends. One end fits within the tapered end of the inlet tube holder whilst the other end is pressed by the compression spring into the gas entry aperture of the ion box.

IsoPrime-EA User Manual

The tapers on both the ion box and the inlet tube holder are designed so that a line seal is formed between the ceramic inlet tube and the other tapers. This mechanism ensures a self-aligning and gas tight seal for the inlet probe.

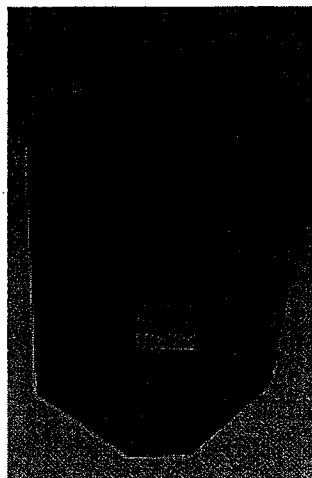


Gas Inlet Probe

Analyser magnet

Permanent magnet

The permanent magnet has an 8mm gap and a field of ~5750 gauss. It is suitable for use with a mass range of 20 – 70 daltons. Selection of the mass of interest is by variation of the acceleration voltage. The permanent magnet has been optimised to work best when measuring the species carbon dioxide and nitrogen. As source sensitivity increases with acceleration voltage users will experience a loss in sensitivity as they work with higher mass species.



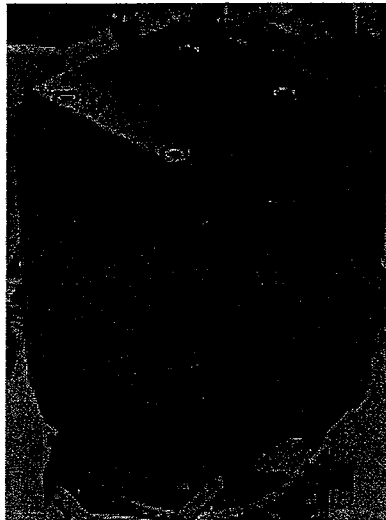
The IsoPrime permanent magnet

Electromagnet

The electromagnet is designed to operate with a current of 1-5A. The jaws of the electromagnet have been designed to provide full coverage of the ion path when working with widely separated masses, e.g. mass 2 & 3. This also provides a wide focal plane on which may be mounted any required combination of collectors.

The magnet power supply is mounted underneath the electromagnet and is controlled via electronics built into the system controller.

The magnet current is fully adjustable from the computer over the range 0-5A with 16-bit resolution. This provides the IsoPrime with a mass range of 0 - ~100 daltons.



The IsoPrime electromagnet

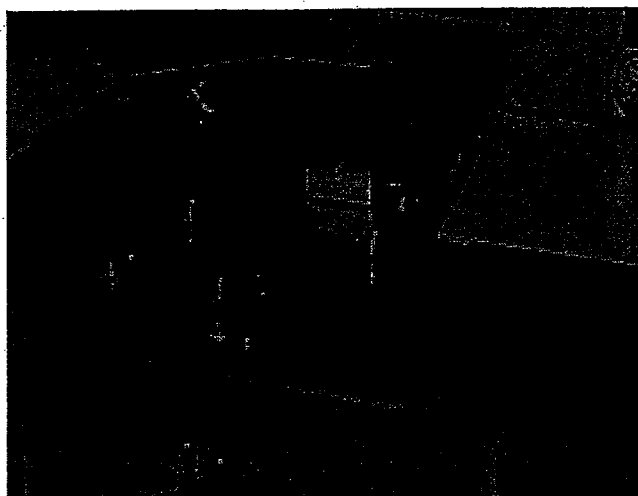
Both magnets are mounted on the same design carriage. A location plate has three ball bearing castors fitted through it. Mounted on the thread protruding behind the castor is a brass nut that engages in the base of the magnet.

The magnet sits on the three brass nuts and the whole carriage is free to roll about the support plate. The magnet is clamped into position using a bolt from beneath the support plate. The bolt may be engaged in two positions, away from the analyser housing (for magnet removal/replacement) or towards the analyser housing (the operating position). The hole in the forward position is sized to allow the magnet to be moved over the normal adjustment range without removal of the bolt.

When the magnet is in the correct position it may be clamped securely by tightening the bolt. Two adjusting screws provide fine adjustment of the magnet position, one to the left of the magnet and one in front of it. These two screws provide location in the X and Y directions. Rotation of the magnet is constrained by an adjustable bracket on the location plate.

The z-height, pitch, and yaw of the magnet are provided by adjustment of the three brass nuts.

Once the magnet has been positioned correctly and the adjusting screws moved into position, the magnet may be removed from the analyser and returned to the original position with ease.



Magnet carriage

Analyser housing

The analyser housing is manufactured from stainless steel. The grade of steel used ensures low remanent magnetic fields within the analyser.

The housing is rectangular in section with two end flanges sealed with viton seals. The two end flanges carry the source and collectors. The flight tube is integrated into a cut-out in the side of the housing.

The flight tube section is constructed from 1mm thick stainless steel plate to allow the jaws of the magnet to sit around it.

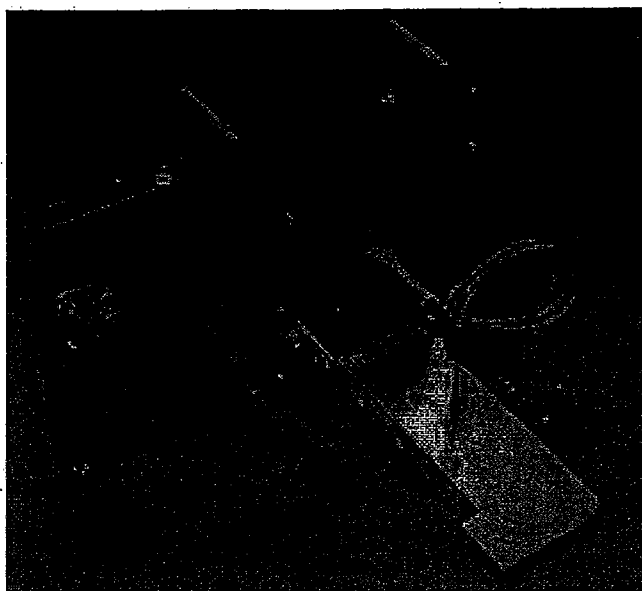
Baffles are fixed within the analyser to restrict unwanted ions from reaching the collectors. The baffles are readily removable for cleaning during normal maintenance.

The arrangement of the integrated flight tube in the housing allows an unobstructed focal plane. This allows a number of masses to be measured simultaneously.

Collectors

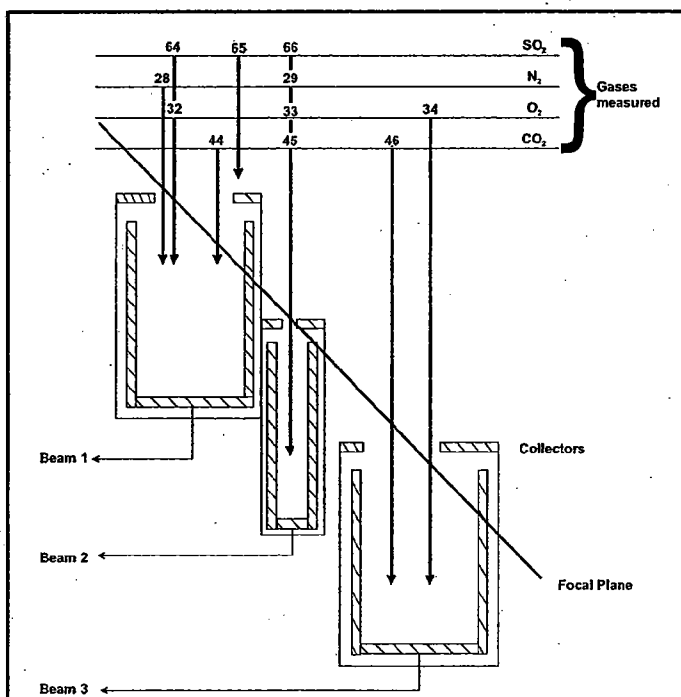
The IsoPrime uses the universal collector depicted below for all the gases except for Hydrogen. This provides three individual collectors each with different width resolving slits.

The narrowest accepts the ion beam from the minor 1 isotope and is referred to as the axial collector. The narrow slit gives relatively good resolving power and rejection of stray ions. The slit is wider than the width of the ion beam and therefore the scanned peak shape is 'flat-topped', this reduces errors in the ratio measurements due to small drifts in ion beam position.



Universal collector construction

The larger outer collectors have slits that are approximately three times wider than the axial collector does, they accept the ion beams from the major and minor 2 isotopes. This accommodates the variations in the ion beam separations for the different gases (e.g. CO_2 , N_2), without the need for moveable collector assemblies.



Collector schematic (beam mask)

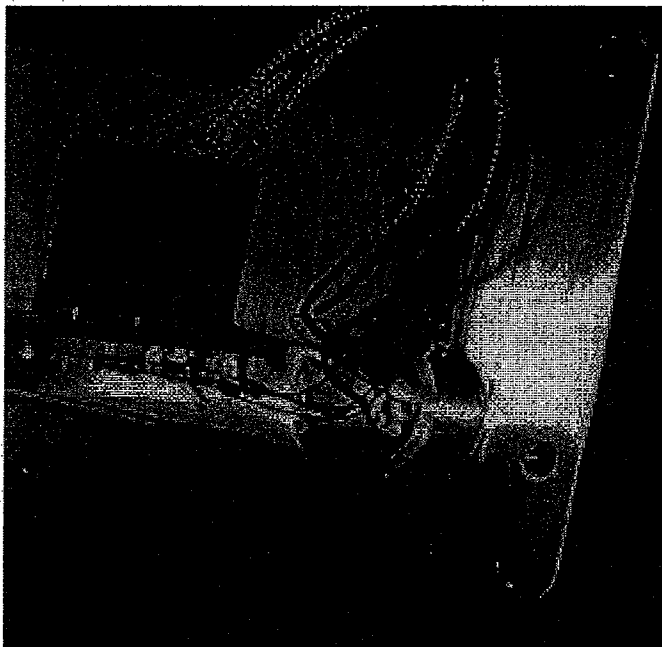
Electron suppressor plates are fitted to each collector and are maintained at approximately -43V . The suppressor is mounted between the resolving

IsoPrime-EA User Manual

slits and the Faraday buckets in order to minimise ion drift between adjacent collectors and also to reject any secondary electrons generated by ion bombardments.

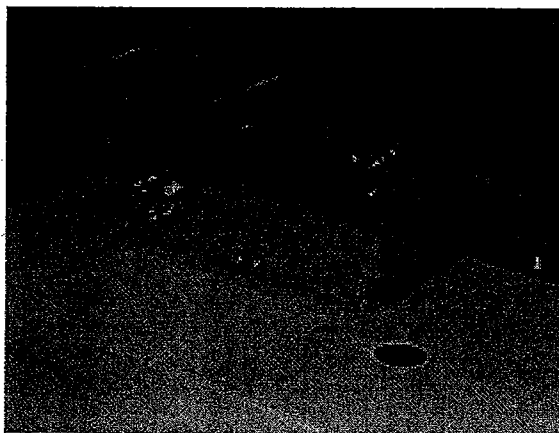
Each individual collector is mounted on a mounting plate that is in turn fixed to the collector flange. The mounting plate extends along the focal plane of the analyser and allows collectors to be mounted at any point along the plane.

The signal leads from the collectors are double screened to avoid any pickup from unwanted ions and connect to the collector feedthrough inside the collector feedthrough shroud.



Collector housing internal view showing collector wiring

The connections to the collectors and the suppressor supply are made via a single nine pin feedthrough flange. Eight of the pins are available for connection to collectors and the central pin takes the suppressor supply.



Collector feedthrough outside view

Vacuum system

Overview

The pumping system is designed to produce the clean high vacuum required for reliable high precision stable isotope analyses. It also provides the high pumping speeds essential for the high gas flow rates encountered in continuous flow applications.

Analyser pumping system

The analyser pumping system is composed of an Edwards RV3 rotary pump which provides the backing for an Edwards EXT250 turbomolecular pump.

The rotary pump is connected to the IsoPrime by a 2m length of flexible hose. This allows the rotary pump to be positioned at a convenient position behind and below the IsoPrime.

The backing line from the rotary pump is connected to a foreline trap filled with activated alumina. An isolation valve allows the rotary pump to be isolated from the turbomolecular pump, enabling the rotary pump to remain operating whilst the analyser is vented. This ensures that moisture from the air is not adsorbed onto the foreline trap, which will prolong pumpdown times.

The isolation valve and foreline trap is mounted on the rear of the IsoPrime for easy access. For ease of maintenance, the foreline trap is fitted with a vent valve enabling replacement of the packing without venting the analyser.

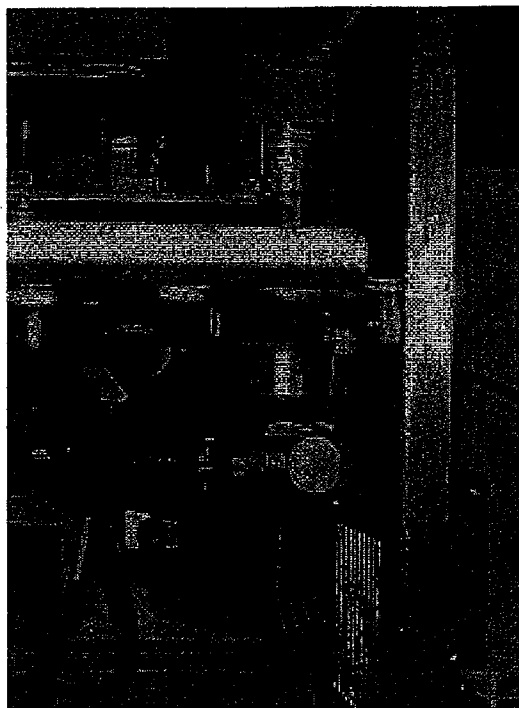
The rotary pump is fitted with an oil mist filter to prevent unwanted emission of pump oil into the laboratory atmosphere.

An automatic vent valve is fitted to the turbomolecular pump. This ensures that even under conditions of power failure the chances of rotary pump oil entering the analyser are minimised. A filter is fitted to the vent valve to stop unwanted particles entering the vacuum system during the venting process.

The turbomolecular pump is cooled by fans fitted into the case of the instrument. Two fans are mounted on the rear panel of the IsoPrime. One, mounted directly behind the analyser turbo pump and electronics case, draws air into the case and provides cooling for the analyser turbo pump. The other fan, mounted at the other end of the case, extracts air from the case to ensure an airflow from the right of the case to the left.

All the components of the turbomolecular pump are controlled by an EXC100L controller mounted on the left of the IsoPrime.

The source control electronics are disabled until the turbo pump reaches full speed.



Analyser Pump Layout



Caution: Routine maintenance is essential to reliable performance of the IsoPrime. A good, clean vacuum is fundamental for the correct operation of an isotope ratio mass spectrometer. Following the manufacturers recommended service schedule will reap its reward in continued trouble free operation.

Please refer to the maintenance section and the manufacturers manuals for service procedures and intervals for all components of the pumping system.

Pressure Measurement

Penning Gauges

An active inverted magnetron (AIM or Penning) gauge is fitted to the ion optics housing to monitor the pressure of the system. The AIM gauge is mounted on a NW25 elbow beneath the collector end of the housing.

The AIM gauge is controlled and monitored from the electronics unit, which is located at the right of the IsoPrime cubicle. The AIM gauge is enabled as soon as the turbo pump reaches ~95% speed. The software displays the pressure on the computer (see later sections of this manual for details).

Protection against overpressure of the AIM gauge is provided by monitoring the turbo pump speed. Should the speed of the turbo pump drop below 95% the AIM gauge is disabled.

Note: For information on the Penning Gauge control see later sections of this manual for details.

Pirani gauges

An APG S NW16, active Pirani gauge is used to measure the pressure in the analyser pumping backing line. This gauge is located adjacent to the turbo pump roughing port and reads at all times that the electronics unit is powered.

Vacuum protection

The pressure measuring devices, in conjunction with the vacuum control provide vacuum protection for the Penning gauge and ion source.

Any condition that results in overpressure will cause the ion source power supply and Penning gauge to switch off.

Electrical and Electronics



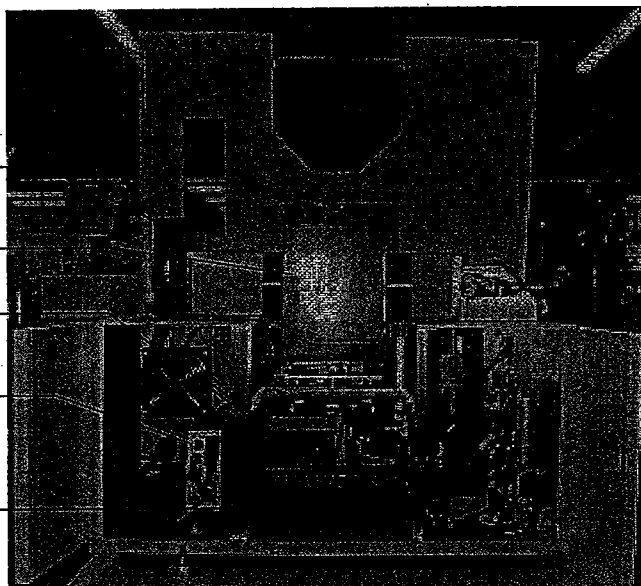
Warning: All electrical and electronic units can cause injury, so care must be taken.

Overview

This section gives a brief description of the functions of each of the IsoPrime's electronic units.

Unit Locations

Head amplifier
Permanent magnet
Mains distribution
Turbomolecular pump
controller
System controller



IsoPrime layout (front view)

Roughing isolation valve
Foreline trap vent valve
Rating plate
Mains inlet
RS232 computer
connection



IsoPrime layout (rear view)

The electronic units that may be fitted in the IsoPrime comprise the following:

IsoPrime-EA User Manual

- System Controller
- Turbomolecular pump controller
- Head amplifier
- Magnet Supply
- Bakeout power supply

The minimum configuration for an IsoPrime is the system controller, turbomolecular pump controller and a head amplifier. Other units may be present depending on the configuration of the instrument.

System Controller

The system controller is located inside the cubicle behind the door on the right hand side. This location allows the closest connection of the high voltage supplies to the source; this arrangement imparts the greatest protection to the system against flashover.

The system controller controls all the electronic systems of the instrument. The controller is based around a dedicated Motorola 68000 microprocessor. This provided all the control necessary for the system to operate the instrument.

The data system controls the instrument by communicating with the system controller via an RS232 serial link. There is a constant exchange of instructions and data between these two computer systems.

Incorporated within the system controller are the power supplies and control electronics for the complete instrument. Control functions supported are listed below.

- Active gauge interface for two gauges (pirani and penning).
- Power supplies for instrument cooling.
- Valve control for continuous flow interfaces.
- Electromagnet control.
- Head amplifier power supply.
- Four channels of VFC acquisition for ion beams
- Three additional channel of data acquisition for FID and TCD signals.
- TCD power supply.
- GC and EA start/stop signals.
- Turbomolecular pump controller logic.
- Bakeout control.
- Control of all the source parameters and high voltage supplies

System firmware is written in 'C' and is based upon the well-proven firmware used within the Optima and Isochrom instruments.

IsoPrime-EA User Manual

The systems controller has been designed to be easily removable by the customer and offers the possibility of exchange by the customer on site, thus precluding the expense of an engineer visiting.

Source and High Voltage Supplies



Warning: These units generate the voltages and currents required to drive the gas source. Their function is built into and controlled by the system controller.

High Voltage Supplies

High voltage is produced by three supplies located within the system controller unit. The voltages generated are used to drive the ion source. A total of three supplies are used, one to provide the source HT potential and the other two to provide focus and, by differential offset of the voltage references, half plate steering.

The high voltage supplies are referenced to the ground potential of the instrument.

The system controller board also produces the Z steering voltage.

Source Control Unit

The source control unit is built into the system controller. The source control generates other current and voltage supplies required for the operation of the ion source. The whole of the source control is floated at accelerating potential and superimposes its supplies upon this voltage.

Source voltages and currents generated by the source control unit are:

- Filament current
- Trap current
- Electron volts
- Ion - repeller voltage

Source connections

All the source voltages and currents are attached to the source via cables, which connect into the top of the system controller. The source connections and cable are protected by a cover fitted over the end of the analyser housing.

All source potentials are monitored by the systems controller and may be read back and displayed on the data system.

Head Amplifier

The 4-channel amplifier is housed in a rugged aluminium casting. It is secured to the side of the collector housing by two recessed bolts. Location is aided by two dowel posts on the side of the housing. The connection to the collectors and to the secondary electron suppressor is by a 9 pin gold feedthrough. The connectors in the amplifier are spring-loaded to ensure good contact.

Internally the amplifier is divided into 2 compartments, each housing a printed circuit board. The upper board (the "analogue board") is responsible for amplifying the collector currents by converting them into a

IsoPrime-EA User Manual

voltage (0V to 10V). Prominent on this board are the 4 low temperature coefficient resistors. The resistors used are:

Low Mass 2: 5×10^8 ohms (not used)
Low Mass 1: 5×10^8 ohms
Axial: 5×10^{10} ohms
High Mass: 1×10^{11} ohms

As the maximum output voltage of each amplifier is 10V, the maximum source current can be calculated:

Low Mass 2: 2E-8A (not used)
Low Mass 1: 2E-8A
Axial: 2E-10A
High Mass: 1E-10A

A unique feature of the amplifier is that it digitises the amplified signal prior to transmission. This is performed by voltage to frequency converters on the analogue board. The conversion is scaled such that the full-scale 10V output from the amplifier is represented by a frequency of 1 MHz.

The second printed circuit board is called the 'digital board'. Its function is to turn the digitised signal from the amplifier board into pulses of red light which are then transmitted to the System Controller via optical fibre. The use of a fibre optic link makes the transmission of data immune to external electrical interference.

The system controller is responsible for counting the number of light pulses that arrive at its VFC card in each 100mS integration period. Some simple arithmetic shows that a full-scale signal is equivalent to 100,000 "counts" in each period.

The power supply for the amplifier and the secondary electron suppresser is connected to the digital board via a DIN plug.



Caution: The amplifier contains very static sensitive components. It should be handled carefully. Before removing the amplifier, turn off the source to prevent voltage build-up on the feedthrough pins. Short the nine feedthrough pins to the collector housing (using a screwdriver) before fitting the amplifier back on. Only when the amplifier has been refitted and connected is it safe to switch the source back on.



Caution: Never touch any of the components inside the amplifier, as contamination may produce small tracking currents that could have a serious affect performance. Never attempt to clean the head amplifier components, this will almost certainly result in the same effect..

Electromagnet supply

The electromagnet supply is located directly underneath the electromagnet and is mounted within the electromagnet support. The unit is powered from the electronics mains circuit. It contains a raw DC supply and a programmable DC-DC converter under the control of the system controller.

Turbomolecular Pump Controller

The turbo pump controller (Edwards EXC100L) is situated on the left of the instrument. It is connected to the system controller for speed readout and on/ off control. For more information about the controller refer to the manufacturer's instructions.

Mains distribution

The power supply for the IsoPrime is provided by a single IEC connector located on the rear left of the instrument. This power input is connected to an IEC distribution block mounted on the rear panel of the IsoPrime. Connection leads from this block provide power to the various electronic units fitted to the instrument.

The mains circuit is protected by a double fused IEC inlet connector. Fuse ratings and type are clearly indicated on the rating plate of the instrument.



Caution: Under no circumstances should electronic units other than those fitted within the IsoPrime cubicle be connected to the distribution block. Failure to observe this precaution may result in overloading of the mains supply system.

See the fault-finding section for details of all fuses in the system.

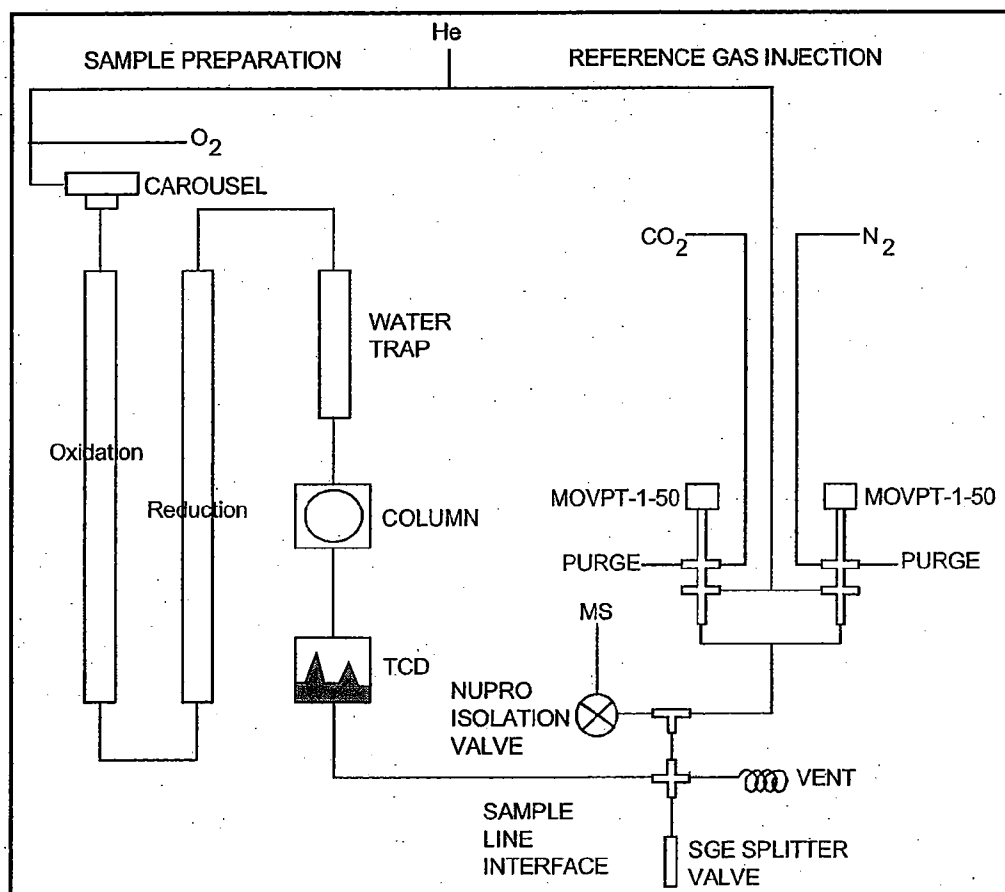
The EA interface

Overview

The EA interface system comprises a number of different units, each designed to perform a specific function within the sample preparation-analysis cycle. A figure of the system is shown below.

The units are:

- The elemental analyser for the combustion of samples and the separation of resulting gas species N_2 , CO_2 , SO_2 .
- The IsoPrime interface for establishing the conditions under which sample and reference gases are presented to the mass spectrometer.
- The IsoPrime mass spectrometer previously described.



The Elemental Analyser

The most common type of elemental analyser fitted to the system is the CE Instruments NA2500. Most options associated with this analyser can be implemented. A full description of the NA2500 along with a list of available options are contained within the manufacturer's product manual supplied with the instrument.

Reference Gas Injector

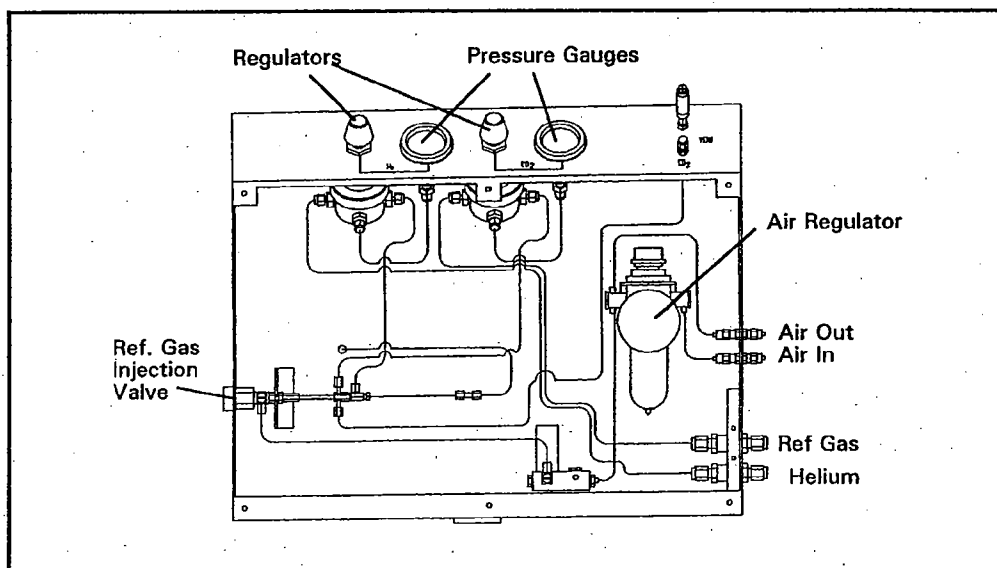
The reference gas injector is designed to introduce stable pulses of pure gas of known isotopic composition into the analyser at constant intensity, for the purposes of system calibration. There are two forms of reference gas injector:

- Single gas injector capable of delivering pulses of one species of reference gas
- Dual gas injector capable of delivering pulses of two species of reference gas

Both types of injector require supplies of each species of reference gas and of Helium carrier gas. Details of the quality of gases required are given in the site planning guide found in the Appendices section of this manual.

Single gas injector unit

The schematic for the single reference gas injector is shown in the figure below.

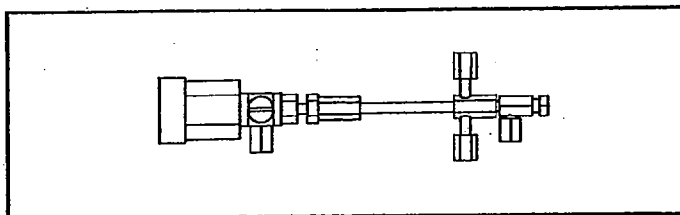


Single Reference Gas Injector

The system comprises an outer single reference gas box assembly in which are enclosed:

Reference Gas Injection Valve

The reference gas injection valve releases (mnemonic is RG) reference gas into a continuous stream of Helium. The gas flow from the reference gas injector is sampled by the mass spectrometer. A schematic of the reference gas injection valve is shown in the figure below.



Reference Gas Injection Valve

The valve body is an SGE MOVPT 1/50 normally closed needle valve actuated by compressed air (see manufacturer's product information). At the base of the valve sits a back-drilled, SGE VG-GVF 003 ferrule, into which is fitted a 70mm length of 25 μ m ID fused silica capillary. The end of the piston plunger shaft acts on the top face of the back-drilled ferrule. When the compressed air is on, the end of the shaft is lifted off the back-drilled ferrule, allowing reference gas to flow down the 25 μ m capillary to mix with the continuous stream of reference Helium introduced through the SGE VG-MFT 16 tee piece attached to the end of the MOVPT valve. The gas mixture passes through an 8cm length of stainless steel 1/16x0.006" capillary, and flows into a length of stainless steel 1/16x0.030" capillary into which are inserted the reference gas and standby fused silica capillaries. Instructions on the fitting of these fused silica capillaries are given in the Maintenance section of this manual.

The Gas Network

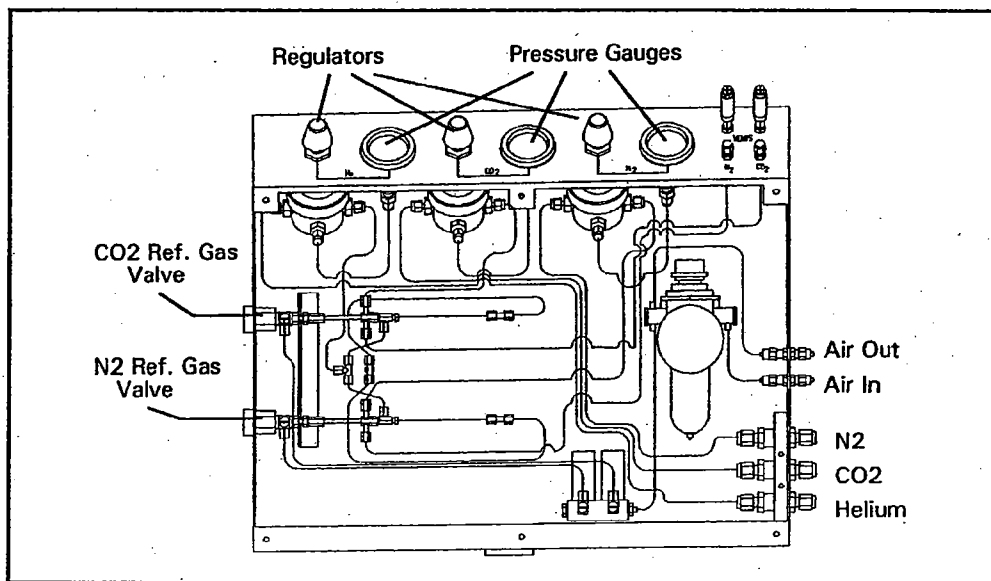
The reference gas and reference Helium pressures are controlled by regulators mounted on the top of the reference gas injection box. The Helium and reference gas sources are connected to the rear of the reference gas injection box via an in-line filter. The gases flow directly to manual pressure regulators, 0 - 10psi for the reference Helium and 0 - 30psi for the reference gas. The gas pressures are read from pressure gauges mounted in the top of the reference gas injector box. The reference Helium flows to the exit capillary via the reference gas injection valves. The reference gas flows to the tee assembly at the end of the SGE MOVPT 1/50 valve and hence to an exit vent situated at the top right of the reference gas injection box. The rate of flow from the reference gas vent is controlled by an adjacent SGE BMCV-1 needle valve.

The Compressed Air System

The reference gas valve (mnemonics are RN for N₂ and RG for CO₂) is actuated by compressed air delivered from a suitable supply through a bulkhead fitting situated on the rear of the reference gas box. The compressed air is unregulated and must be supplied suitable regulated and filtered according to the details laid down in the site requirements guide. The compressed air supply passes to the reference gas valve via a normally closed Predyne valve. The firmware address and mnemonic for this valve varies depending on whether the reference gas injector is designed for Nitrogen or Carbon Dioxide. A 25-way D-type plug allows the reference gas box to be connected to the system controller for operation of the valves. Activation of the Predyne valve sends compressed air, at 55 psi, to the reference gas valve. This lifts the piston plunger in the reference gas valve and allows reference gas to be introduced to the Helium stream, and hence to the mass spectrometer.

Dual gas injector unit

The dual reference gas injector unit is similar in design to the single type. However, it is capable of dispensing two gas species, usually N_2 and CO_2 . The schematic for the dual reference gas injector is shown in the figure below.



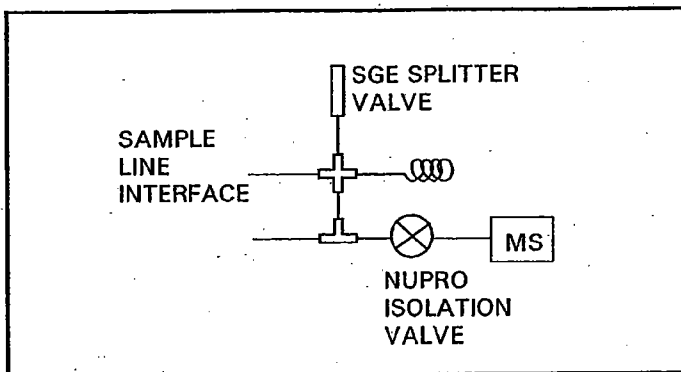
Dual Reference Gas Injector

Sample/ reference line interface

The sample/ reference line interface for the EA comprises the following components:

- EA outlet connection fitting.
- Sample splitter valve assembly.
- Sample and reference fused silica capillaries.

The sample flow from the elemental analyser is passed to the splitter assembly via the outlet connection fitting. The SGE splitter valve allows a small but variable amount of the sample flow (normally approximately 0.3ml) to pass to the mass spectrometer. The reference gas flow is introduced via a tee on the base of the splitter valve assembly. A fused silica restriction capillary (2m of 75 μ m ID) is used to restrict the amount of gas entering from the reference flow to account for approximately 1/10 of the total flow into the mass spectrometer. Both the sample and reference gas flows then pass through the Nupro isolation valve into the mass spectrometer ion source.



The Gas Distribution

The following gases are required for the operation of the EA interface. Details of the necessary quality for these gases are given in the site planning guide in the Appendices at the end of this manual. The gases required are:

Elemental Analyser

- Helium Carrier gas for the EA.
- Oxygen Required for the sample flash combustion process.
- Air (compressed) For operating the sample carousel.

Reference gas injection

- Helium Carries reference gas to the mass spectrometer
- CO₂ Isotopic reference gas.
- N₂ Isotopic reference gas.
- Air (compressed) For operating the valves.

Note

The gas cylinders should be located as close to the instrument as possible and connected using 1/8" stainless steel tube.

Utility gas requirements.

Note: Gas pressure in units of bar.

| | Helium | Oxygen | Air | CO ₂ | N ₂ |
|-------------|--------|--------|-----|-----------------|----------------|
| NA 2500 | 4 | 4 | 3 | n/a | n/a |
| Ref gas box | 4 | n/a | 3 | 4 | 4 |
| Purity /% | 99.999 | 99.999 | DRY | 99.999 | 99.999 |

Operational Overview

The EA interface is based around the principle of interfacing the effluent flow from a standard elemental analyser directly into the ion source of a mass spectrometer in order to provide isotopic information in addition to the quantitative information already available from the elemental analyser.

CE Instruments NA2500 Elemental Analyser

Mode of operation exactly the same as described in system operating instructions. The EA functions as an on-line prep system for the production of N₂, CO₂, SO₂, H₂O from solid or liquid samples.

Samples are loaded on to the carousel and sequentially dropped into the combustion furnace. A pulse of oxygen promotes a flash combustion of the sample at 1800°C. The resulting gases are swept through a reduction furnace, converting oxides of nitrogen to nitrogen. Water of combustion is removed by a chemical water trap. Nitrogen, Carbon Dioxide and Sulphur are then separated spatially in time by a packed GC column. The amount of each species is measured by a thermal conductivity detector connected to some type of integrator. The time taken for any analysis is dependent on - the length of the GC column, its temperature and the flow rate of the helium carrier gas.

Interface

Designed to sample the effluent helium stream from the NA2500, introduce a portion of the stream in a safe manner to the mass spectrometer ion source and enable the introduction of a reference gas pulse for isotopic comparison.

The sample line flow of about 100ml per min coming from the elemental analyser is connected to an SGE splitter valve. The function of this valve is to control the amount of sample line helium actually flowing into the mass spectrometer source. In normal operating mode, this would be about 0.3ml per min giving an analyser pressure of 3E-6 mbar. Altering the flow rate into the source allows more or less sample into the source. Once the interface split has been set the SGE valve should, if at all possible, not be altered due to the length of time required for it to settle to a steady split. The flow from the SGE valve is connected to a tee into which is brought, the reference gas capillary from the reference gas box. The capillary is pushed 6cm past the tee toward the mass spec prior to tightening the ferrule.

The reference capillary consists of 2M of 75um ID fused silica quartz capillary, one end of which is pushed 30cm up the inside of the 1/16" by 0.030" s/s capillary coming from the reference gas box. This capillary samples the reference gas stream in an open split configuration and on its own would produce a pressure in the analyser of about 5E-7mbar. The amount of reference helium or reference gas sampled depends on the length and ID of the capillary and the relevant gas pressures as set in the reference gas box.

Reference gas injection

The function of this component is to provide reference gas pulses for introduction to the mass spectrometer.

The Autosampler Carousel

Minimum capacity of 50 samples. Can increase capacity to 196 samples by the addition of 3 more sample drums.

TCD Operation.

The EA interface has the ability to measure elemental composition using the TCD of the elemental analyser or the mass spec detector.

Data System

Because of the rapidly changing nature of the PC market it is not possible to give a specification of the data system used here as it would rapidly be obsolete.

In general terms the data system will have sufficient power to run the OS/2 operating systems required for the software and have at least one serial port for communications to the mass spectrometer.

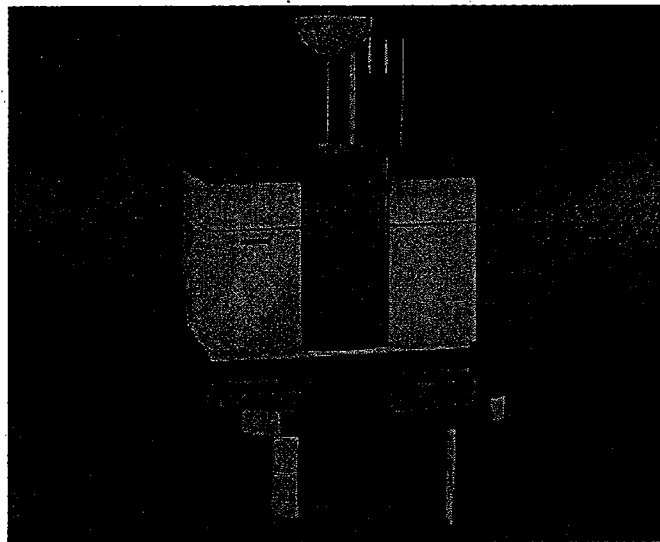
A dot matrix printer will also normally form part of the data system.

The data system is connected, by a serial cable to the rear of the mass spectrometer.

Please refer to the manuals supplied by the manufacturer for more details about the data system.

IsoPrime EA User Manual
Code No 6666588
Issue 1a

Section 5



User Interface



IsoPrime - EA User Manual

| | |
|------------------------------------|-----------|
| Overview..... | 9 |
| OS/2 Environment..... | 9 |
| Computer Start Up..... | 9 |
| Automatic Start-up | 9 |
| StartUp Problems | 9 |
| Computer Shut Down | 10 |
| Why? | 10 |
| How? | 10 |
| OS/2 Desk Top | 11 |
| Desk Top Environment | 12 |
| Using the mouse..... | 12 |
| Clicking..... | 12 |
| Dragging..... | 13 |
| Windows | 13 |
| Moving Between Windows | 15 |
| Dragging A Window..... | 15 |
| Sizing A Window | 16 |
| Icons..... | 16 |
| Iconising A Window..... | 16 |
| Restoring an Iconised Window..... | 16 |
| Using The Keyboard..... | 16 |
| Selecting Menus | 16 |
| Moving the menu selection bar..... | 16 |
| Dialogue Boxes | 17 |
| Special Keys | 17 |
| Menus..... | 18 |
| Menu Tick (On/Off) | 18 |
| Sub Menu Arrow | 18 |
| Grey Menu Items | 18 |
| The User Interface | 19 |
| Dialogue Box..... | 19 |
| Radio Button..... | 19 |
| Check box..... | 20 |
| Push Button | 20 |
| List Box | 20 |
| Entry Field..... | 20 |

IsoPrime - EA User Manual

| | |
|--|-----------|
| Combo Box | 20 |
| Scroll Bar | 20 |
| Tab | 21 |
| Greyed Functions | 21 |
| Printing..... | 21 |
| Multitasking | 21 |
| IsoPrime - EA Operating Environment | 22 |
| Screen Layout | 22 |
| Main Menu Bar | 23 |
| Choosing Commands Using The Mouse | 23 |
| Choosing Commands Using The Keyboard | 23 |
| The Monitor Window | 23 |
| Vacuum Gauges | 24 |
| Ion Beams | 24 |
| The Message Window..... | 24 |
| The Mimic Diagram Window | 25 |
| Valves | 25 |
| IsoPrime - EA Menus..... | 26 |
| Inlet | 26 |
| Mass Spec | 26 |
| Tune Source | 26 |
| Peak Jump | 29 |
| Source On..... | 30 |
| Source Off..... | 30 |
| Enable Hall Probe | 30 |
| Disable Hall Probe | 30 |
| Penning On..... | 30 |
| Penning Off..... | 30 |
| Scan..... | 30 |
| Edit Param File..... | 31 |
| Scan..... | 31 |
| Run Scan Param File..... | 35 |
| Scan Display Window | 36 |
| Scan Display Window Menu Bar..... | 36 |
| Graph | 36 |
| Cursor | 38 |
| Lines..... | 39 |
| Window..... | 39 |

IsoPrime - EA User Manual

| | |
|-------------------------------------|----|
| Scan..... | 40 |
| Tests..... | 40 |
| Peak Quality..... | 40 |
| Identify Peak..... | 41 |
| Mass Calibration..... | 42 |
| Peak Centre..... | 42 |
| Analysis..... | 43 |
| Method Setup..... | 44 |
| Gases Overlay..... | 46 |
| Jump Mode..... | 48 |
| Sequences Overlay..... | 48 |
| Valves Overlay..... | 49 |
| Acquisition Overlay..... | 50 |
| Analysis overlay..... | 51 |
| Extended parameters window..... | 53 |
| Report Overlay..... | 56 |
| TCD Overlay..... | 56 |
| Analysis..... | 57 |
| Single Run..... | 57 |
| Edit Autorun →..... | 59 |
| 'Load AutoRun File' dialog box..... | 59 |
| Autorun Batch Dialog Box..... | 61 |
| Autorun Stop..... | 64 |
| Parameter File Edit..... | 65 |
| Batch DP Option..... | 66 |
| User..... | 67 |
| Level 1..... | 67 |
| Level 2..... | 67 |
| Supervisor..... | 67 |
| Log In..... | 68 |
| Log Out..... | 68 |
| List User..... | 68 |
| Create New User..... | 69 |
| Modify User..... | 70 |
| Delete User..... | 71 |
| View Own Details..... | 72 |
| Change Password..... | 72 |
| Program..... | 73 |
| Run..... | 73 |

IsoPrime - EA User Manual

| | |
|--|-----------|
| Terminate | 74 |
| Hold | 74 |
| Continue | 74 |
| Config | 74 |
| Monitor | 75 |
| Gauge | 77 |
| Help | 78 |
| Help Window | 78 |
| Help for Help | 79 |
| Keys help | 79 |
| Help index | 79 |
| About | 79 |
| Language | 79 |
| DP - Manual Data Processing | 80 |
| Introduction | 80 |
| Data Storage | 80 |
| Accessing the DP software | 80 |
| Loading Data | 81 |
| The DP main window menu bar | 81 |
| File | 82 |
| Load Data | 82 |
| Load Params | 82 |
| Save Params | 82 |
| Del Params | 82 |
| Init Params | 82 |
| Export Data | 82 |
| Edit | 83 |
| Select All | 83 |
| Deselect All | 83 |
| New Point | 83 |
| SD | 83 |
| Select Peak | 83 |
| View | 83 |
| Ref | 84 |
| Sample | 84 |
| Background 2/1 | 84 |
| Background 3/1 | 84 |
| Zero | 84 |
| Iso Shift 2/1 | 85 |

IsoPrime - EA User Manual

| | |
|---|------------|
| Iso Shift 3/1 | 85 |
| Calculate | 85 |
| Re Analyse | 85 |
| Full Analysis | 85 |
| Default Analysis | 85 |
| Report | 86 |
| Results | 86 |
| Raw Data | 86 |
| Parameters | 87 |
| Integration Parameters | 88 |
| General | 93 |
| TCD DATA | 94 |
| Edit Zeros/Gas | 96 |
| Status | 96 |
| Help | 97 |
| DP Graph Window Menu Bar | 97 |
| Graph | 97 |
| Cursor | 99 |
| Lines | 100 |
| Window | 100 |
| Parameter Files | 101 |
| Autorun Procedure Settings Parameter File | 101 |
| Constants Parameter File | 102 |
| Current Rack Parameters | 105 |
| General Parameters | 105 |
| HP Rack Parameters | 105 |
| Retention Times Parameter File | 106 |

IsoPrime - EA User Manual

Overview

The 'user interface' is the way the user interacts with the mass spectrometer from the computer system. This section of the manual will therefore detail the essential aspects that are needed to accomplish this, however it will be necessary to read the OS/2 manuals supplied with the instrument to complement this section.

Its purpose is therefore to guide the user through the features necessary in running the IsoPrime - EA software, and in conjunction with the context sensitive HELP facility, enable the user to learn the basics of the software that will lead to instrument operation in the next section of this manual. The screen displays are used to familiarise the user with the appearance of the user interface.

OS/2 Environment

This section of the manual cannot serve as a full guide to all the powerful features provided with the OS/2 operating system, but is intended to give the user the basic features required to start running the IsoPrime - EA software package.

Note: PLEASE READ THE OS/2 MANUALS SUPPLIED WITH THE SYSTEM.

The IsoPrime - EA is controlled largely through the use of a 'mouse', pull down menus, and graphical representations of the system called 'icons'. There is no need to learn complicated keyboard sequences, and the system is easy to operate. Some people may prefer to use the Keyboard: there are simple 2 key alternatives to virtually all "mouse" operations. "Hot Keys" give simple access to frequently required options. The Keyboard is of course used in any case to enter data, such as sample names, reference gas compositions, to set integration times etc.

Computer Start Up

OS/2 preserves the layout of the Desktop even when switched off. This means the user no longer have to move icons to the top of a list to get them to start automatically - anything open at shutdown will be opened again next reboot. As well as leaving applications open at shutdown the user can place applications in the start-up folder, these will then be started each time at reboot.

Automatic Start-up

To start an application automatically simply drag the application icon (e.g. EA) into the Start-up Folder (in the OS/2 System Folder on the Desktop). For example to start-up the system clock automatically: - Open the OS/2 system folder. Open the System Setup and the StartUp folders. Now pick the Clock icon from System Setup and Copy it into the StartUp folder. This application will now be run each time the system reboots.

StartUp Problems

As OS/2 will always start applications that were running when it was Shutdown, it is possible to get problems. However, it is possible to disable the automatic program startup. To do this reboot the machine and when the white screen first appears press and hold the left Ctrl, left Shift, and F1 keys. Hold them down until the icons appear on the desktop.

Computer Shut Down

Why?

To speed up access to commonly used files and applications OS/2 now uses a Caching system. A Cache is an area of memory set aside for the storage of data. This means that some files are accessed and left open, stored in the Cache. Any reference to these files accesses the copy in memory (in the Cache), thus saving the time to search and load from disk. When the computer is switched off, or re-booted, the contents of this memory disappears, possibly losing data or corrupting files. Wherever possible before turning the machine off or rebooting the user should perform the Shutdown operation. This will clear the Cache saving any changes and closing files.

In addition to clearing the Cache shutdown will close down all the applications and folders which are open. OS/2 keeps track of the layout of the Desktop, where the folders are and what applications are running. Next time the user boots into OS/2 these will be opened automatically and the Desktop will look exactly as it was at Shutdown. This means ALL folders/programs open at Shutdown will be opened again. The more applications open the slower the system runs so any folders that are not wanted to be opened next time should be closed BEFORE performing the Shutdown (see Window List section).

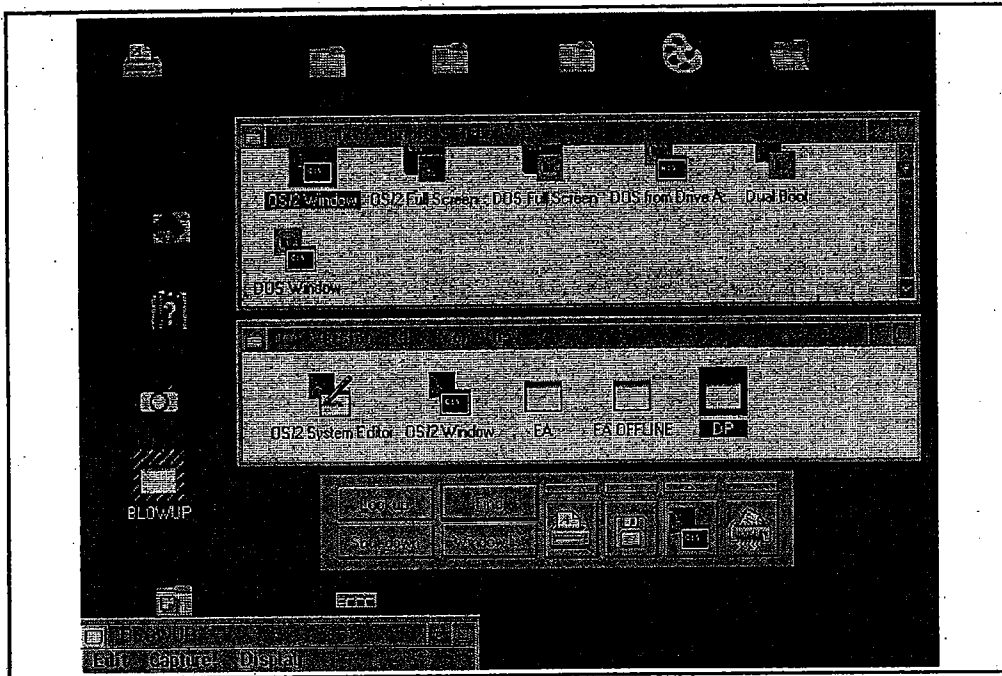
Note: Some applications will ask for confirmation to close down - this ensures that data is not lost in applications mistakenly left open.

How?

On the desktop, move to an area away from any icons. Bring up the Desktop menu (single click Right mouse button), and select shutdown. Wait for the message Shutdown Completed. The user can now reboot or switch off the computer.

OS/2 Desk Top

On starting the computer the first screen to be seen is the OS/2 Desk Top, an example of which can be seen below. From this, access to any of the applications can be achieved by using the mouse to click on the various icons displayed.



Desk Top Environment

Each application started runs in its own window and a number of application windows can be open on the desk top at any one time. The screen can therefore be used by a number of Windows, which each display information related to a specific area of the software.

One helpful way of thinking about this is to regard the windows as sheets of paper on a "DESKTOP" represented by the whole screen display. Just like paper on a desktop, the windows can be picked up and moved around, put on top of each other etc. Unlike sheets of paper, some of the windows can be shrunk or expanded, either to fill the entire screen, or to occupy a minimum amount of screen space.

The entire IsoPrime - EA program is itself a Window. If the user wishes to run another program, such as a spreadsheet, as well as the mass spectrometer, the IsoPrime - EA window can be sized to suit the display needs. It can even be shrunk to an Icon, and run in the background while the whole screen is available for other applications.

Using the mouse

The mouse is a device incorporating a tracker ball, which moves a pointer around the screen. The mouse pointer is the 'hand' that picks things up and moves them around on the desktop. The mouse also has two buttons at the top that can be depressed (clicked). The left mouse button is the most used button. It is used for selecting, resizing, dragging, copying objects and starting applications etc., within the IsoPrime - EA software. The right button is used for moving objects and setting their attributes.

Clicking

When the pointer is over the menu item or icon that you wish to select, a press or "click" on the left mouse button will highlight that item.

In the case of the main menu bar, clicking on one of the menu options will 'pull down' a further menu. Use the mouse to highlight the option wanted. A further click will then action that option. It is possible to do this more quickly by doing two button presses in quick succession. This is called 'double clicking'.

More detail of Clicking is given below:

- **Single Clicking (with the left mouse button)**
Single clicking on an object (icon, window, or menu) will select it, highlight it. This means that the object now has input focus (i.e. it will respond to anything typed at the keyboard and the other objects won't) or is just selected (depending on what type of object it is).
- **Double Clicking (with the left mouse button)**
Double clicking on a program icon will start that program, (double clicking on a valve icon will open that valve or close it).
Also it is worth noting that double clicking on the small icon in the top left corner of a running application will generally close the application (this is the same as single clicking here and selecting close).
- **Single clicking (with the right mouse button)**
When the user single clicks on a menu name this will bring up a menu list (can also use the left mouse button). The other place this is used is when closing down the system.

- **Double Clicking (with the right mouse button)**
This has no effect.
- **Pressing both buttons together**
This only has effect if the mouse pointer is currently pointing at the desktop, it will bring up a list of all of the programs currently running on the system (see Window List).

Note: Use right mouse button to move icons, etc. and the left button to drag windows.

Dragging

To drag an object (essentially move it), position the mouse pointer over the object and press and hold down the right mouse button, now when the mouse is moved the object will move with the mouse pointer. Care must be taken however when dropping the object.

The most important uses of dragging are:

- **Window Positioning:** any window with a Title Bar can be repositioned by dragging on the title bar.
- **Window Sizing:** a window can be expanded or contracted by dragging on its borders. Dragging on the corners will size in two dimensions simultaneously.

Note: The mouse pointer changes to a double-headed arrow to show that it is possible to resize.

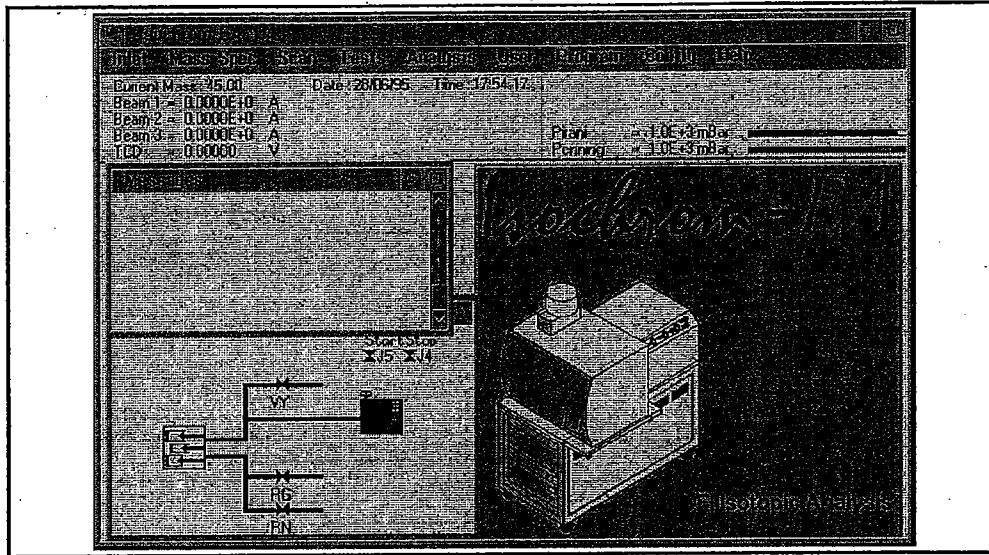
- **Using Slider Bars.** Slider bars are used to scan through long amounts of text in text windows (e.g. Help Index), and in two important parts of the IsoPrime - EA software: Message list and source tuning.

Note: It is also possible to move an object to another location by selecting the move option from the System Menu.

Windows

All Windows have the same features and can therefore be discussed generally, however the IsoPrime - EA software is being used in this example. Windows can have other windows running in them, with the highest level known as the 'Application Window' and the inner windows known as 'Child Windows'.

The entire IsoPrime - EA software runs as an 'Application Window'.



At the top is the 'Title Bar'. At the left end of this is the 'System Menu'. At the right end are the 'Minimise' and 'Maximise' boxes. The System Menu has the following features, which appear as a menu list by clicking once in the System Menu (please beware that a double click will close the IsoPrime - EA software down, double clicking in most System Menus will close that particular application down):

| | |
|-----------------|----------|
| Move | Alt+F7 |
| Size | Alt+F8 |
| Minimize | Alt+F9 |
| Maximize | Alt+F10 |
| Hide | Alt+F11 |
| Close | Alt+F4 |
| Window List | Ctrl+Esc |
| Arrange Windows | |

- Restore**
 If an application has been resized then the clicking in restore will restore the window to its default / original size.
- Move**
 This enables the window to be moved around the screen, the window follows the mouse until the left or right mouse button is clicked (see also dragging a window).
- Size**
 This is used to change the size of a window, by selecting size and moving the mouse pointer to one of the window borders that border will be picked up and move with the mouse until the left mouse button is clicked (see also sizing a window).
- The next three commands can either be found in the menu or in the top right hand corner of a window as icons for maximising, minimising and restoring.

- **Minimise**



This is used to minimise a window to an icon. When a window is minimised the particular 'Minimise' icon for that window is placed in the 'Minimise Icon Viewer' folder.

- **Maximise**



This is used to maximise a window to its largest possible size.

- **Restore**



The restore icon can be used to 'restore' the window to its original size, for example, if the user were to maximise, or indeed re-size the IsoPrime - EA window and then wished to return to the original window size then this can be achieved with the 'Restore' icon.

- **Hide**

This is not used by the IsoPrime - EA software and hence is 'Greyed Out' (see section on Greyed Out).

- **Close**

This is used to close the application window down, ending any programs running from that window.

- **Windows List**

This opens the 'Window List' dialogue box which lists the active programs (also possible using <CTRL. .ESC>).

- **Arrange Windows**

This is used to arrange the windows 'Child Windows' in the 'Application Window', hence tidying the screen layout.

Moving Between Windows

It is often needed to move between windows to view or change information.

To move between windows when the window wanted is visible:

- Click anywhere in the window that is required.

The software activates the window and brings it to the front if there are other windows covering it. The active window has a coloured border and title bar, inactive windows are monochrome. This window now has 'input focus'.

Dragging A Window

Windows can be moved to any location on the screen by dragging them to a different location. This is achieved by:

1. Move the mouse pointer to the title bar of the window to be moved.
2. Press and hold down the left or right mouse button.
3. Drag the window in the direction for it to be moved.
4. When the window is in the new location, release the mouse button.

Sizing A Window

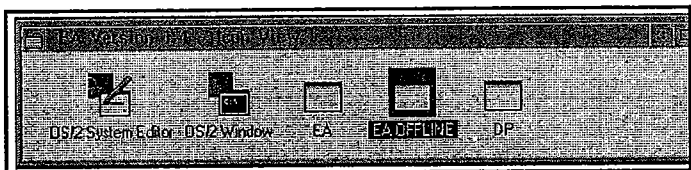
The windows can be made smaller or larger, by moving the borders of the window in or out.

1. Move the mouse pointer to the border of the window (the pointer changes shape to a double arrow when it is in position).
2. Press and hold down the left or right mouse button.
3. Drag the border in the direction desired.
4. When satisfied with the new size, release the mouse button.

Note: Going to the corner of a window enables the window to be re-sized in two directions at once.

Icons

Icons are small picture symbols used to represent either graphical objects (valves, etc.), or windows. An icon which represents a window or application has the name of the application beneath it. When a window is minimised it is represented by its Icon. Within some windows, notably the 'EA Child Window', icons are used to represent real objects, such as valves, etc. (clicking on these icons can produce an action or open a dialogue box).



Iconising A Window

When any of the child windows are minimised, they will appear in the ICON AREA at the bottom of the main IsoPrime - EA window. Windows that can be iconised are the Message Window, the Scan Window, the Data Acquisition Window and the EA-IRMS Window.

Restoring an Iconised Window

A double click on the icon and the window will be restored to its previous size. If the iconised window had a System Menu, a single click on the icon with the right mouse button will access a menu with options to restore, reposition or maximise the window.

Using The Keyboard

Selecting Menus

Each of the Main Menus has an underlined letter. Keying this letter whilst holding down the ALT key will select that menu. Items within the menus can also be selected by using the underscored key.

Note: The System Menu is selected by ALT SPACE.

Moving the menu selection bar

The selection bar can be moved with the cursor keys. When the desired option is highlighted (shown in black), it can be actioned by pressing the return key.

Dialogue Boxes

Movement between entry fields in Dialogue boxes is achieved by using the TAB key and cursor keys. The buttons in dialogue boxes such as Yes, No, Help, etc., are controlled via pressing ALT and the underlined letter or using the cursor keys to give focus to the key needed to select and then pressing the return key.

Special Keys

Certain keys have special applications, or have been allocated as "Hot Keys" which speed up software operation without needing to use the mouse. These are listed and explained below.

- **F1**
The F1 key will open the Help Window. If detailed Help is available for the part of the software that is being used, the appropriate Help Text will be displayed.
- **Esc (Escape)**
This will remove the Help Window and most dialogue boxes without causing any action.
- **Enter (or carriage return)**
Usually this will action the OK or Run Button in a dialogue box.
- **Tab**
This key is used to move between different fields in Dialogue Boxes.
- **Cursor Keys**
These are used to move within dialogue box fields, and between Menus and Commands.
The arrow keys give a fine adjustment, the Page Up and Page Down Keys a coarse step.
- **Alt**
Holding the Alt key down while keying the Underlined letter in a Menu or Command will select that option.
- **Control**
The control Key is used in the same way as the ALT key to access certain commands quickly:
 - Ctrl + C - performs a Peak Centre
 - Ctrl + I - opens the Identify peak dialogue box
 - Ctrl + J - starts a peak jump
 - Ctrl + Q - performs the peak quality routine
 - Ctrl + R - Runs an analysis
 - Ctrl + S - Stops a scan and closes the Scan Window
 - Ctrl + X - Opens the Scan Parameters library

Menus

Menus are a list of functions that can be accessed by selecting the menu item from the list. After selecting (clicking on the menu item or using one of the special keys) some action will take place or the software will require some further information from the user or further menus will be revealed.

Note: If a menu or button has a '...' it means that there is a dialogue box activated by this menu or button, rather than starting a task.

The menu lists will be discussed in further detail in later sections of this manual, however there are some features of menus that need to be discussed at this stage.

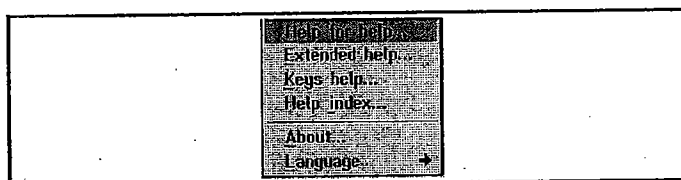
Menu Tick (On/Off)

When a tick appears at the left-hand side of a menu item then that function is selected, when the menu item is again selected the tick will disappear hence de-selecting the menu item function. This therefore indicates the application of a toggle type function on the menu item. An example this can be found on the 'Zoom In' menu item in 'Scan Display Window' (see later for details of Scan Display Window).



Sub Menu Arrow

If an arrow appears on the right hand side of a menu item then that item will have a sub menu. Therefore another menu will be below the original menu. An example of this can be seen in IsoPrime - EA software 'Help' menu option 'Language'.

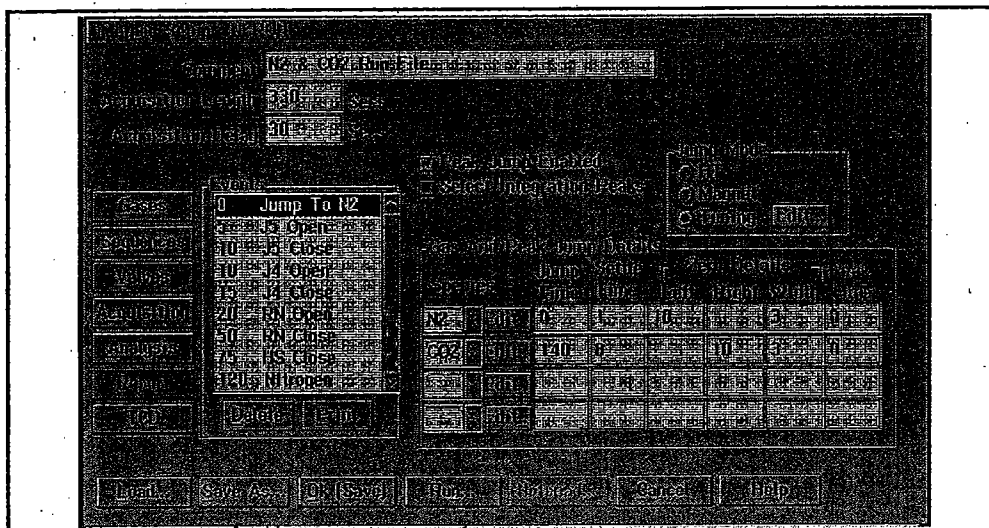


Grey Menu Items

If a menu item appears grey instead of black, then that menu item cannot be accessed at that time. An example of this can be seen in the System Menu when the window is at full screen the 'maximise' option is greyed out, as this has no function when the window is already maximised.

The User Interface

This section of the manual describes the input controls available in OS/2, however the examples will be taken from the IsoPrime - EA software. The main example will be the Method Set-up Dialogue Box as this shows most of the required controls.



Dialogue Box

Dialogue boxes are windows through which the user can answer requests for data or provide information. They may contain any of the controls to be discussed later in this section, for example: entry fields for text or numerical input, push buttons for Help, To OK or Cancel an action, and 'Check Boxes' or 'Radio Buttons' to select or de-select options.

Dialogue boxes generally do not have System Menu and can therefore not be minimised, etc. This generally means they take precedent over any other function and must be answered or cancelled prior to other user operations.

The operational sequence facility available in the software enables the user to create dialogue box questions for instrument control.

Radio Button

These have an on / off function if they are on their own, however the more usual example is as part of a list within a box, where if one function from the list is chosen, the other functions are de-selected (there can be various boxes each containing a different set of radio buttons). In the example, in the "Fit Type" compartment, if 'Polynomial' is chosen then 'Spline' is turned off, and visa versa. A radio button is selected by clicking on the circle next to the item description, on is indicated by a solid black dot in the centre of the circle.

Check box

These have a toggle function where the option is selected or not. Selection is shown with a tick or a cross in the box and is achieved by clicking on the box next to the item description. An example of this appears in the "Method Set up" window shown previously. A tick appears on the left-hand side of "Bknd Subtraction", indicating that background subtraction will be performed when this method is used.

Push Button

These perform a command when selected, for example saving data, calling the help file, opening another dialogue box, etc. The default Push Button is emphasised by a heavy border and is selected by simply pressing the keyboard Return key.

List Box

This gives the user a list of options to be chosen from. These appear in alphabetical order and can be selected with a double click or by a single click followed by a command (Push Button). If the list is longer than the box the list can be scrolled up and down using the scroll bar at the side of the list. The items are selected with a single click and activated with a double click.

Entry Field

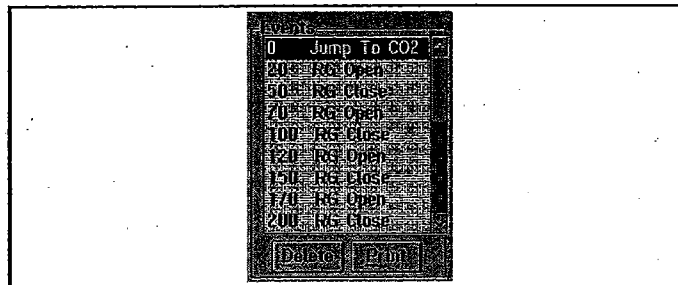
This is an area set aside to accept user-inputted data into the dialogue box, for example file names, data, etc.

Combo Box

This is a combination of an entry field and a list box. When the arrow at the side of the box is clicked on a list box appears from which the various options can be selected as with a list box. Alternatively if the user knows the option name then this can be typed directly into the entry field of the combo box.

Scroll Bar

This is sometimes known as a slider bar and can be horizontal or vertical. The purpose of the Scroll Bar is to move a list. An example of a scroll bar appears in the "Method Setup" window. The complete list of "Events" can be examined by moving the Scroll Bar up and down.



Clicking on the arrows will move the slider a small amount, clicking to the side of the slider moves the slider a medium amount and if large moves are required then drag the slider by clicking and holding down the mouse button and moving in the direction required.

Tab

It is possible to move between entry fields in Dialogue boxes by using the TAB key and cursor keys.

Greyed Functions

If a function is in grey text rather than black text, then that function is not available at this moment. For example if the datalogger is disabled then the datalogger options are not required and are therefore greyed out.

Printing

When a job is sent to the printer then a print job object will be created within the printer folder. This print job object represents your print job in the queue. It is possible to view any queued jobs by double clicking on the icon.

Note: If the spooler is disabled then jobs are sent directly to the printer and they will NOT appear in the printer folder.

There are a number of ways that a job can be printed:

1. The application that is running generates a print job.
2. Use the Print facilities available in the software.
3. Use the 'Print' command from the DOS or OS/2 command line.
4. Drag an object and drop it onto a printer object.
5. Position the pointer over the dialogue box or window that is to be printed and press the "Print Screen" key to dump the dialogue box or window to the printer.

Multitasking

The multitasking capabilities extend the power of the software enormously.

These are of two levels of multitasking.

First, each program that runs under OS/2, such as the IsoPrime - EA software, may be "multithreading" i.e. many processes can be active simultaneously. For example the user can be performing an automatic analysis whilst preparing samples on another part of the system, carry out data acquisition simultaneously with any other activity, tune the ion source whilst scanning etc.

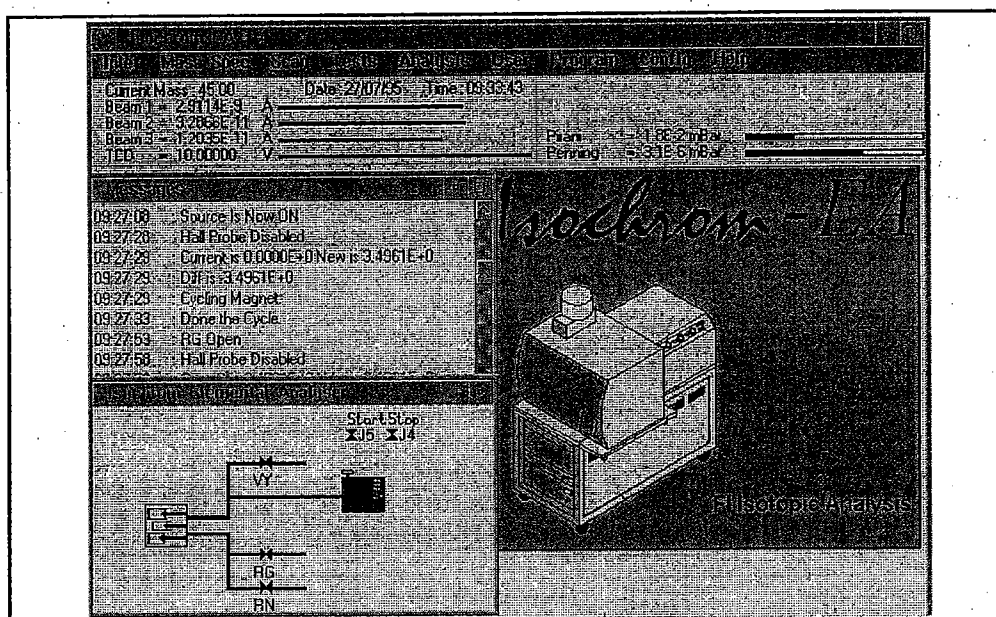
Secondly, the system has true "multitasking" i.e. more than one program or "application" can be running at any given time. For example the user can be running the instrument at the same time as analysing data with a spreadsheet and whilst writing a report with a word processor.

IsoPrime - EA Operating Environment

This section of the manual will specifically describe the IsoPrime - EA Operating Environment. Some of this section will however have been described as examples in the previous section on the OS/2 Environment.

Screen Layout

The entire IsoPrime - EA software runs as an 'Application Window' with various 'Child Windows' running inside it. The application window and/or the child windows can be resized to fill the entire screen area, or they can be resized/repositioned to occupy any amount of the screen area. For example, the screen area used by the IsoPrime - EA can be reduced if you are using a spreadsheet application. The window can be minimised and run as an icon if desired.



The Application Window and its child windows form three main areas.

Below the Menu Bar is the Monitor Window, which contains numerical and graphical readouts of the ion beams and vacuum gauges. This cannot be moved, resized, or iconised.

Below the Monitor Window is the main work area of the IsoPrime - EA software. The Message Window appears in this area. Additional windows, and dialogue boxes, etc. will appear here as the software is used.

At the bottom of the screen are windows that contain representations the appropriate preparation system valves, etc. fitted to the machine (EA, etc.). These are user definable and will depend on which inlet and/or preparation systems are fitted. They can be minimised to icons if required.

Main Menu Bar

The diagram below shows the Main Menu Bar from which gives the operator gains access to the rest of the pull down menus available in the IsoPrime-EA. The Main Menu Bar is therefore the primary menu.

Choosing Commands Using The Mouse

For example, to choose the Mass Spec, Tune Source command:

Click the Mass Spec menu option in the Menu Bar.

The Mass Spec menu appears with a list of commands.

1. Click the desired command, in this case, Tune Source
2. After the command has been clicked, the menu disappears. The command will either perform an action or display a dialogue box to get more information from the user.
3. Click the cancel button to cancel a dialogue box.

Choosing Commands Using The Keyboard

Using the same example as above to choose the Mass Spec, Tune Source command with the Keyboard:

Notice that there is an underlined letter in each of the menu names. For the Mass Spec menu, the underlined letter is an M.

1. Press ALT and hold down and type the letter M.

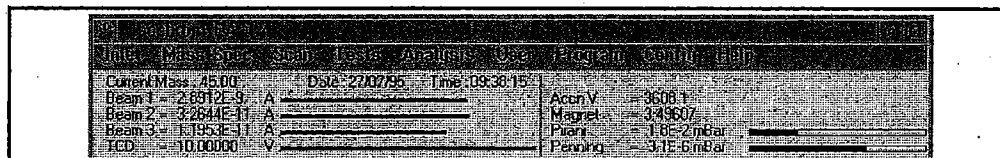
The Mass Spec menu appears.

Some of the commands in the menu have an underlined letter. For the Tune Source command, the letter T is underlined

1. Type the letter T to choose the Tune Source command.
2. Press ESCAPE to cancel a dialogue box.

The Monitor Window

The monitor window is situated just below the Main Menu Bar at the top of the screen. Unlike the other windows, it cannot be resized or iconised. It is used to display important information about the mass spectrometer. The right hand half shows the vacuum gauge status (Numeric and Graphical Displays), and the left hand half shows the Ion Currents and TCD signal size (Numeric and Graphical Displays), as well as the information on the Current Mass (mass in the axial collector) and the date and time.



Vacuum Gauges

The right hand half in our example shows the readings from one Pirani gauge and from a Penning gauge.

Note: This area can display up to 5 gauges that are user definable.

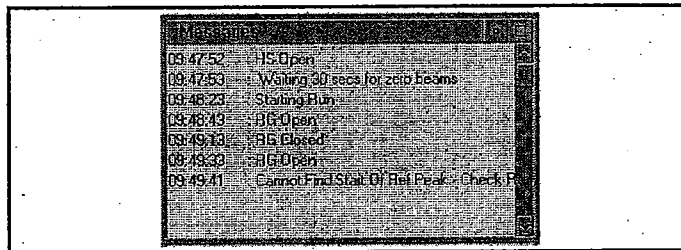
The pressure in millibars is shown next to a horizontal bar that gives a graphical readout of the pressure. The bars are colour coded: green indicates pressure in the normal safe range, yellow a warning of high pressure, and red indicates an unsafe high pressure.

Ion Beams

The signal from 3 Faraday Detectors is shown as Amps to the left of a horizontal bar that gives a visual impression of the beam intensity. The bars are on a logarithmic scale.

The Message Window

The message window appears in the left hand half of the central screen area. It can be resized or iconised if required, but has no System Menu and therefore cannot be closed down. This window is used to display text messages from the software. It is possible to send messages to the text window from other programs.



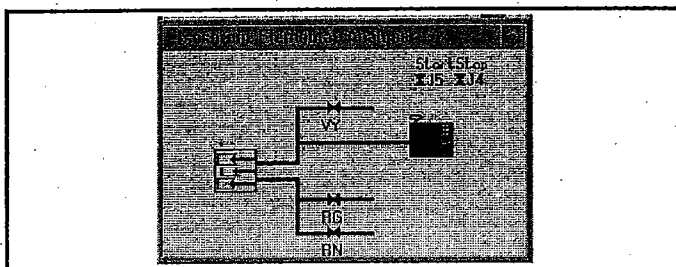
The message consists of a time for the message followed by the text. After the display portion of the window is full, it is possible to step backwards and forwards through the messages via the scroll bar at the right hand side of the window. This window forms a commentary on the operations performed which provides a record of all events since the program was first booted up.

The Mimic Diagram Window

The Mimic Diagram window normally appears at the bottom of the screen area. It can be resized or iconised if required, but as with the message window it has no System Menu and therefore cannot be closed down.

This window is used to display the graphical representation, made up of icons, of the sample preparation system. The preparation system can be extended or modified by the user to allow for expansion of the system.

Some of the icons are interactive, allowing operation of them by pointing the mouse at them (the mouse pointer will change shape when correctly positioned over the icon) and clicking once with the left mouse button. This will bring up the dialogue box for the icon allowing for example valve operation.

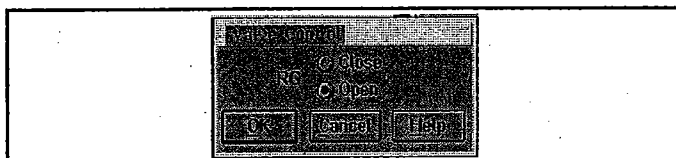


Valves

Use the mouse to point to the desired valve in its mimic diagram window.

Note: the pointer will change size when in range.

Click on the valve icon and the 'Valve Control' dialogue box for that valve will appear, the radio buttons close or open will be selected in the opposite state to the valve.



To toggle the valve either (i) click on the 'OK' push button or (ii) press the return key on the keyboard.

Note: The radio buttons 'Close' and 'Open' can be disregarded as the valves toggle i.e. if the valve is open the next valve dialogue box 'OK' will close the valve and vice versa.

The 'OK' push button appears at the current pointer position, so in practice, a double click toggles the valve.

The Valve control dialogue box also has the following push buttons:

- 'Cancel' will exit the dialogue box without taking any action.
- 'Help' gives information on the operation.

IsoPrime - EA Menus

Across the top of the Main Window is the IsoPrime - EA Main Menu Bar, from which further menus can be selected to control the mass spectrometer. For details of menu selection please see previous section.



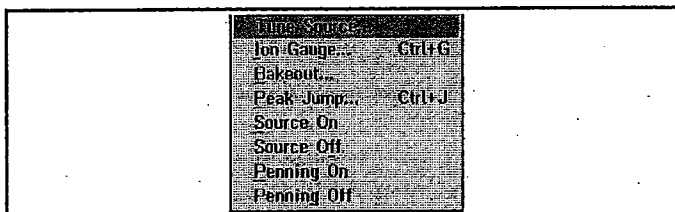
This section of the manual will therefore give a description of all the menus as they appear on the screen.

Inlet

Not normally applicable to EA.

Mass Spec

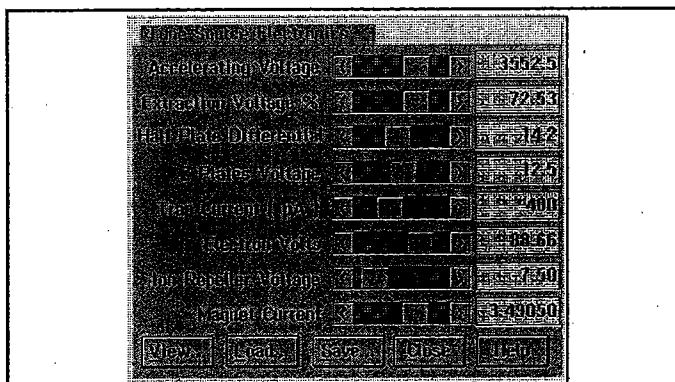
This menu contains all the necessary commands to effectively operate the mass spectrometer.



The options are:

Tune Source

This menu item enables adjustment of the source parameters. It opens the 'Tune Source Electronics' dialogue box, which can be moved around the screen if required.



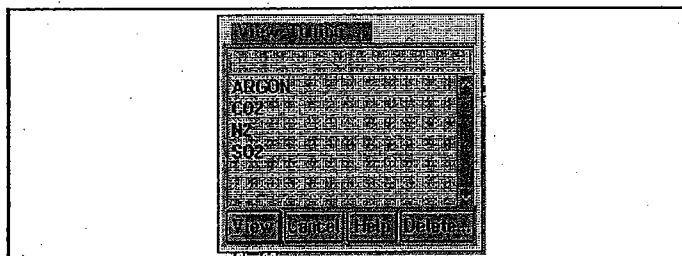
IsoPrime - EA User Manual

- As can be seen the dialogue box controls the following parameters:
- **Accelerating Voltage** between 0 and 5kV
- **Extraction Voltage** between 0 and 100% of the accelerating voltage
- **Half Plate Differential** between -150V and +150V
- **Z Plate Voltage** between -225V and +225V
- **Trap Current** between 0 and 1000 μ A
- **Electron Volts** between 50 and 100 V
- **Ion repeller Voltage** between -15V and +50V
- **Magnet Current** between 0 and 5 Amps

To alter an individual tuning parameter either enter the new value in the entry field next to the source parameter (and click on another box) or use the scroll bar.

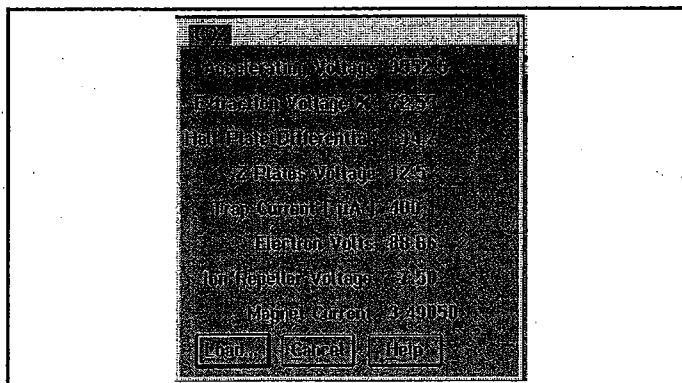
The dialogue box has the following push buttons:

- 'View' allows stored tuning files to be viewed or deleted via the 'View Tuning' dialogue box. This facility therefore allows the parameters to be checked for accuracy and safety prior to loading.



This dialogue box has the following push buttons:

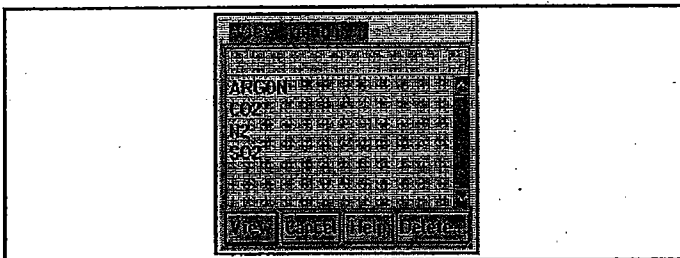
- 'View' loads the source tuning file selected from the list box. This opens the dialogue box below which gives a list of the source parameters for the chosen file.



From here the tuning file can be loaded using the 'Load' push button. The dialogue box can be closed using the 'Cancel' push button or user help can be obtained using the 'Help' push button

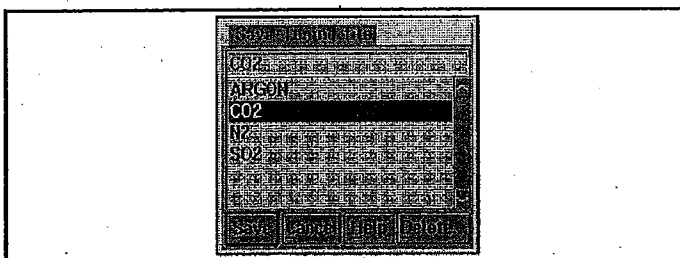
IsoPrime - EA User Manual

- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.
- **'Load'** loads all the voltages and currents indicated by the parameters in this window onto the ion source. Be sure that this action required before the **'Load'** button is clicked!
- **'Load'** opens the **'Load Tuning'** dialogue box, which allows the source tuning files for the various gas species to be loaded (or deleted).



This dialogue box has the following push buttons:

- **'Load'** loads the source tuning file selected from the list box. The tuning parameters in that file are immediately loaded (if unsure then use View first).
- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.
- **'Delete'** allows the user to delete the source tuning file chosen from the list box. A dialogue box is opened to confirm the deletion of the file
- **'Save'** opens the **'Save Tuning file'** dialogue box, which allows a tuning file that has been changed or a new tuning file to be saved.



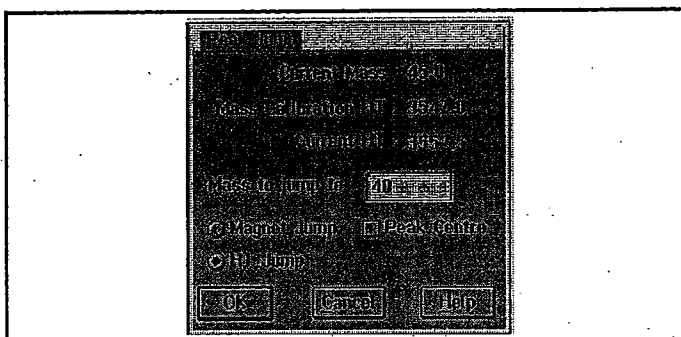
The dialogue box has the following push buttons:

- **'Save'** saves the tuning file under a new file name by typing in a new name in the entry field or can be over written on an existing file name by selecting a name from the list box.
- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.

- **'Delete'** allows the user to delete the source tuning file chosen from the list box. A dialogue box is opened to confirm the deletion of the file
- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.

Peak Jump

This command allows the user to position another mass in the central collector by moving the HT voltage or the magnet current. It opens the **'Peak Jump'** dialogue box, which can be moved around the screen if required. Before a peak jump can take place, the **'Current Mass'** mass must be set from **'Tests'**, **'Identify Peak'**. See the section **Identify Peaks** later in this section.



This dialogue box displays the following:

- **'Current Mass'** (the Identified mass which can be seen in the monitor window)
- **'Mass Calibration HT'** (this is the source voltage at which the mass calibration was taken)
- **'Current HT'** (the source voltage, which for an accurate magnet peak jump must be the same as the mass calibration HT).

The new mass to be focused in the axial collector is entered in the **'Mass to jump to'** entry field. The option is then whether to **'Magnet Jump'** or **'HT Jump'** by selecting one of the radio buttons. After peak jumping the software can be asked to perform a peak centre, by selecting the **'Peak Centre'** check box.

The dialogue box has the following push buttons:

- **'OK'** starts the peak jump routine.
- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.

Source On

This menu option when selected turns **ON** the power to the source electronics. No source potentials are loaded, but the filament is turned on.

Source Off

This menu option when selected turns **OFF** the power to the source electronics.

Enable Hall Probe

This menu option enables the magnet to function with the Hall Probe (i.e. Field Mode), giving a message in the message window 'Hall Probe Enabled'.

Note: This command should be ignored if there is no Hall Probe fitted to the system.

Disable Hall Probe

This menu option disables the Hall Probe (i.e. magnet operating in Current Mode), giving the message 'Hall Probe DISABLED'.

Note: This command should be ignored if there is no Hall Probe fitted to the system.

Penning On

This menu option when selected turns **ON** the Penning Gauge. The command will have no effect if the Penning Gauge is already on.

Penning Off

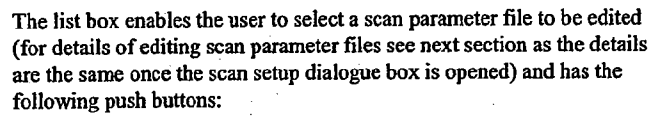
This menu option when selected turns **OFF** the Penning Gauge. The command will have no effect if the Penning Gauge is already off.

Scan

This menu enables the user to perform all the necessary scan functions required, e.g. HT, magnet etc.

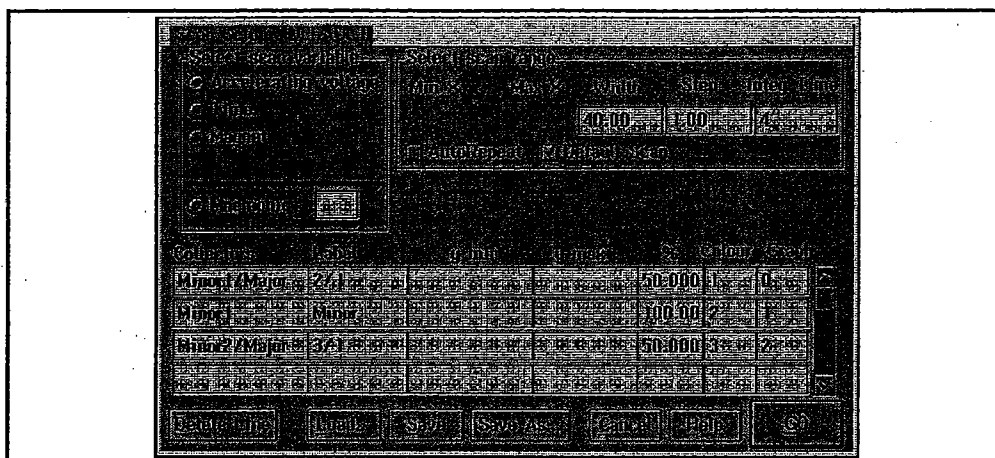


This menu option accesses 'Load Scan Parameter File' list box.



- ## Scan

IsoPrime - EA Menus



The dialogue box will be described in three areas as given below:

'Select scan variable'

- This area contains the following radio buttons that enable the user to choose the X-axis variable to be scanned. (voltage, time or magnet)
- 'Accelerating voltage' allows the amplifier signals to be scanned with respect to the source accelerating voltage, keeping all other parameters at the same conditions.
- 'Time' allows the amplifier signals to be scanned with respect to time (useful when leak checking), keeping all other parameters at the same conditions.
- 'Magnet' allows the amplifier signals to be scanned with respect to the magnet current (or field if the Hall probe is enabled), keeping all other parameters at the same conditions.
- 'Mnemonic' is selected, a source parameter can be scanned by inputting the appropriate mnemonic into the entry field. The mnemonics must be entered as capitals and they are:

| | |
|------|-------------------------|
| EX - | Extraction Voltage |
| HP - | Half Plate Differential |
| ZV - | Z Plate Voltage |
| TR - | Trap Current |
| EV - | Electron Volts |
| IR - | Ion Repeller |

'Select Scan Range'

IsoPrime - EA User Manual

This area controls the X axis variable of the scan. There are entry fields for scan width ('Width'), step size between points ('Step'), and integration time at each point ('Integ Time').

The 'Default Scan' check box is selected (ticked) then the scan range is input in the 'Width' entry field, (this is the total width of the scan, e.g. if when scanning voltage 50V is inputted in the entry field, then the scan will be $\pm 25V$ around the current accelerating voltage). However if the 'Default Scan' check box is not ticked, the 'Width' entry field is no longer accessible. In this case extra entry fields become available 'Min X' and 'Max X', which allow you to select the lower and upper limits of the x values (minimum and maximum to be used).

Notes: If a Time scan is selected, only the 'Max X' entry field is used to enter the duration of the scan in seconds (there is no 'Min X' as time always starts at time zero), and the 'Step' field is used to enter the time step.

If the 'AutoRepeat' check box is selected then the scan will clear at the end of the scan, before starting the scan again (this is particularly useful when peak shaping).

Lower Entry Field (Line Details)

Each line in the bottom part of the dialogue box sets up one trace on the scan (up to a maximum of four).

The fields are:

'Collectors'

Identifies the collector output to plot.

Note: This can be a ratio.

Entries should be made in the exact form shown below

Major

Minor1

Minor2

Minor1/Major

Minor2/Major

There should be no spacing between any of the characters, additionally the first letter of the collector name should be in capital.

- **'Label'**

The text in this field will appear on the screen to label the scan at the right hand edge of the scan window.

- **'y min', 'y max' and '%'**

These 3 fields control the Y-axis scale. The entry fields 'y min' and 'y max' are used to enter specific values (using scientific notation).

By entering a value in the '%' column, the software will automatically scale a ratio or beam intensity within the requested range, according to the intensities measured before starting the scan. **Please note that the entry in the % column to define the scale for a ratio is in units of δ**

- **'Colour' and 'Graph'**

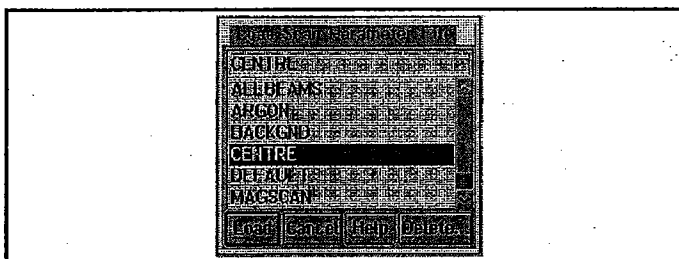
The **'Colour'** entry fields determine the colour in which a line is drawn in the Scan Display Window. A number indicates the colour. The numbers are the same as used in screen messages, the main colours being:

| | | | | | |
|----|------------|---|-----------|----|------------|
| -2 | White | 4 | Green | 10 | Dark red |
| -1 | Black | 5 | Cyan | 11 | Dark Pink |
| 0 | Background | 6 | Yellow | 12 | Dark Green |
| 1 | Blue | 7 | Neutral | 13 | Dark Cyan |
| 2 | Red | 8 | Dark Grey | 14 | Brown |
| 3 | Pink | 9 | Dark blue | 15 | Pale Grey |

The **'Graph'** entry fields allow a number of separate graphs to appear in the Scan Display Window at the same time. You may have up to 4 graphs (entered 0 - 3 in the entry field). If you are plotting beam intensities it may be best to show these all on the same graph (in which case use the same number), to illustrate beam coincidence, but, for example, a ratio is best shown on its own graph, because of the different Y scale.

The Scan Setup dialogue box also has the following push buttons:

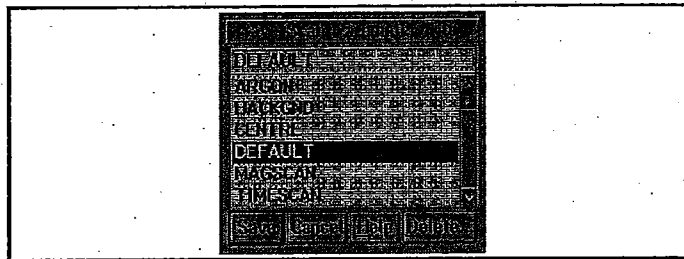
- **'Delete Line'** deletes a selected line from the lower entry details (collector details). The line is selected by selecting any of the entry fields on the line. A confirmation dialogue box is accessed to safeguard against accidental deletion.
- **'Load'** accesses **'Load Scan Parameter File'** list box, which enables you to select a scan parameter file to be loaded and has the following push buttons:



This dialogue box has the following push buttons:

- **'Load'** loads the **'Scan Setup'** dialogue box for the file chosen from the list box.
- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.
- **'Delete'** allows the user to delete the scan parameter file chosen from the list box. A dialogue box is opened to confirm you wish to delete the file ('Yes' or 'No' push buttons).

- 'Save' saves the scan setup file in the existing Scan Parameter File (overwrites the file called up).
- 'Save As' selects the 'Save Scan Parameter File' dialogue box which enables new scan files to be saved and has the following push buttons:

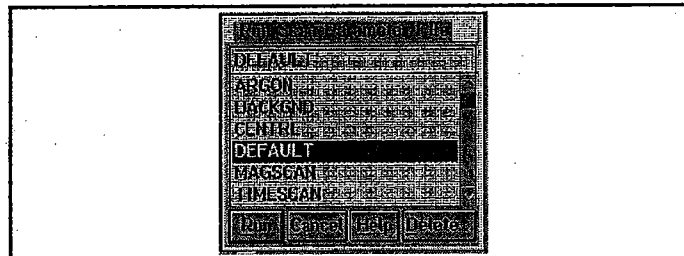


This dialogue box has the following push buttons:

- 'Save' saves the scan setup file in a scan parameter file chosen from the list box or under a new name entered in the entry field.
- 'Cancel' exits the dialogue box without taking any actions.
- 'Help' gives information on the operation.
- 'Delete' allows the user to delete the scan parameter file chosen from the list box. A dialogue box is opened to confirm you wish to delete the file ('Yes' or 'No' push buttons).

Run Scan Param File

This menu option accesses 'Run Scan Parameter File' list box.



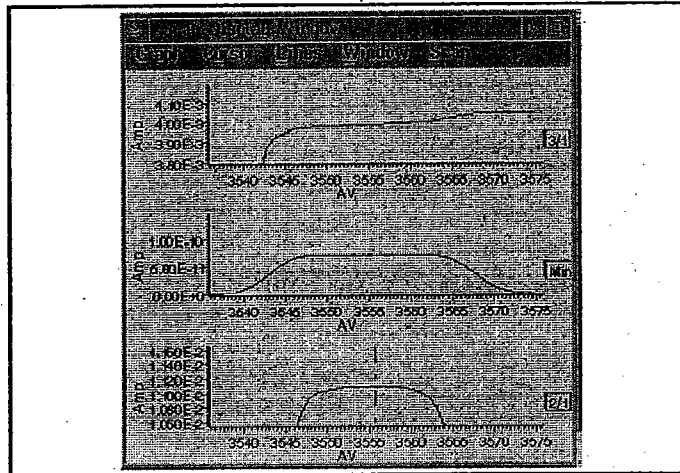
The list box enables you to select a scan parameter file to be run and has the following push buttons:

- 'Run' runs the selected scan parameter file from the list box.
- 'Cancel' exits the dialogue box without taking any actions.
- 'Help' gives information on the operation.
- 'Delete' allows the user to delete the scan parameter file chosen from the list box. A dialogue box is opened to confirm you wish to delete the file ('Yes' or 'No' push buttons).

Note: For details of the Scan Window see below.

Scan Display Window

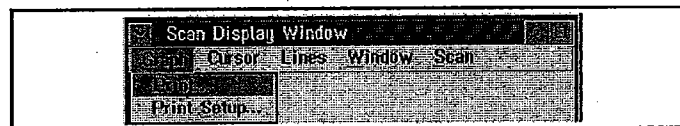
When you start a scan, the 'Scan Display Window' appears in the middle of your screen. It can be resized and positioned to suit your own requirements. The window has its own Menu Bar and will show a graph for each plot you have set up in the 'Scan Setup' dialogue box.



Scan Display Window Menu Bar

The 'Scan Display Window' has the following options on its menu bar:

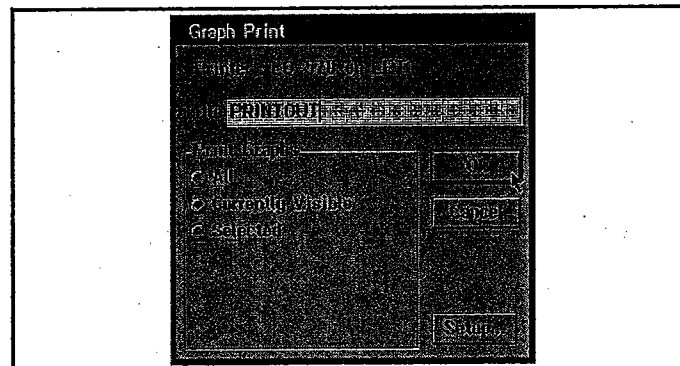
Graph



This menu option has the following commands:

- 'Print'

This menu option opens the 'Graph Print' dialogue box.



This dialogue box allows you to give the print out a title in the 'Title' entry field and to select the graphs to be printed using the radio buttons in the 'Print Graphs' section. The radio buttons are:

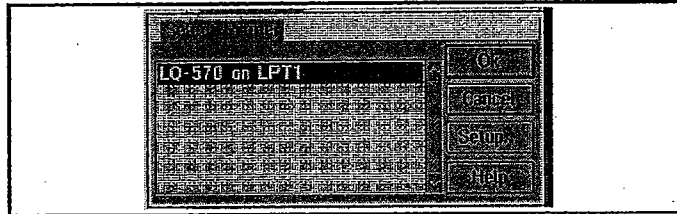
IsoPrime - EA User Manual

- 'All' when selected prints all the graphs selected from scan.
- 'Currently Visible' when selected will print all the graphs in the scan window.
- 'Selected' when selected opens a list box from which graphs to be printed can be selected, by clicking on the required graph title.

The dialogue box also has the following push buttons:

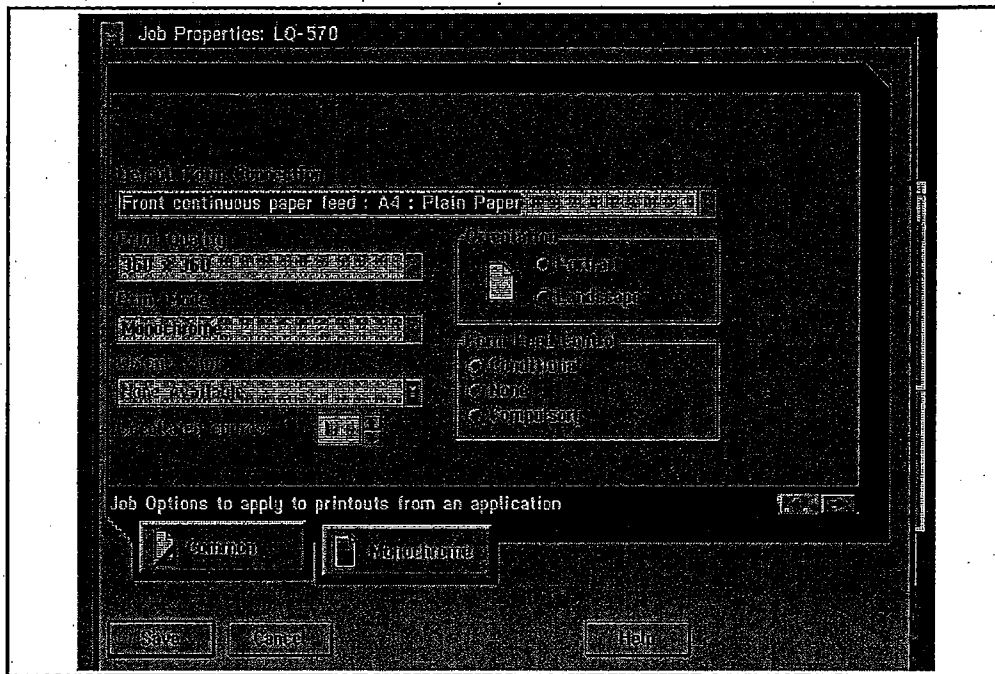
- 'OK' will then start the print out.
 - 'Cancel' push button exits the dialogue box without taking any actions.
 - 'Setup' will open the 'Select Printer' dialogue box as described below.
- 'Print Setup'

This menu option opens the 'Select Printer' dialogue box. This allows the type of printer to be printed to, to be chosen. A list of available printers appears in the list box



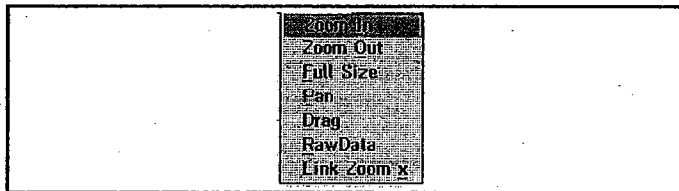
The dialogue box has the following push buttons:

- 'OK' will specify the printer selected in the list box as the preferred printer until changed (by selecting a different printer).
- 'Cancel' push button exits the dialogue box without taking any actions.
- 'Setup' will open the selected printers 'Job Properties' dialogue box, from which the print out details can be edited. For details of the 'Job Properties' dialogue box see OS/2 Manual.



- 'Help' push button gives information on the operation.

Cursor



This menu controls the behaviour of the mouse pointer within the graphical display area. The commands all act with a 'Menu Tick' function and are:

- 'Zoom In'
When chosen, the mouse cursor becomes a magnifying glass symbol. By dragging the magnifying glass over an area of the scan you can examine it in greater detail. If required you can 'Zoom In' in several stages.
- 'Zoom Out'
By clicking on a 'Zoom In' graph after selecting 'Zoom Out', the plot will go back to the size previously displayed prior to a 'Zoom In', this can be done several times until the graph is back to full scale use.
- 'Full Size'
This will return a graph to full scale, simply by clicking on the plot.

- 'Pan'

This is greyed out and has no function.

- 'Drag'

When Drag is selected, you can use the mouse to pick up and move the vertical cursor line used to show the current voltage. This is useful if a new analysis position is required on the flat top.

- 'Raw Data'

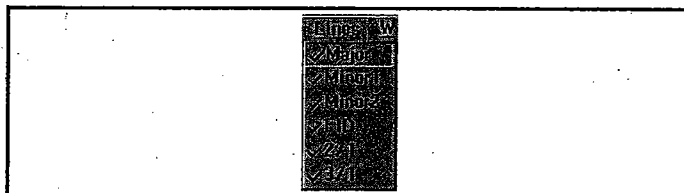
Raw Data mode provides an additional vertical cursor, which can be used in conjunction with the drag facility to examine the raw scan data in a small window at the bottom left of the screen. Only those lines ticked in the lines menu (see below) have their raw data displayed.

- 'Link Zoom'

When selected, the mouse symbol becomes 2 linked loops. Using this, you can link the x scale of two graphs by clicking on them in turn. After adjusting the horizontal scale on a particular graph (graph A), in order to copy this scale onto a second graph (graph B) then:

1. Select link zoom
2. Click on graph A
3. Click on graph B

Lines



This menu contains a list of the lines available for display. The text in the menu is the label you have entered in the Scan Setup dialogue box. By ticking lines on or off from this menu you can simplify the display window.

Window

The commands all act with a 'Menu Tick' function and are:

- 'Cascade'

This default setting will arrange multiple graphs one above the other in the display window (i.e. one column).

- 'Tile'

This option will arrange graphs side by side, as well as above each other (i.e. two or more columns).

Scan



The commands are:

- **'Centre'**
This command automatically centres the voltage cursor (peak centre).
- **'Quality'**
This command performs a peak quality check and sends the results to the printer, (see section on peak quality).
- **'Repeat'**
This repeats the scan once, if auto repeat has not been set.
- **'Halt'**
This command halts a scan while in progress.

Tests

This menu contains a set of commands used to check the status of the instrument, as well as being required for day to day running.



The menu options are:

Peak Quality

This menu option provides a quantitative measurement of the quality of your peak shapes, in terms of flatness, symmetry and resolution. When the **'Peak Quality'** command is chosen a peak centre is carried out (as above) and the resolution, symmetry, and peak top flatness are measured of any peak found within the range of the centre scan, these are then output to the printer.

Note: If the peak quality of all the collectors are required then the other beams need to be added to the centre scan procedure.

Identify Peak

This opens 'Identify Axial Peak' dialogue box, which allows you to identify the mass in the axial collector (i.e. the mass presently in the axial collector or the mass you are going to move to the axial collector).

This is particularly important as this sets the 'beam mask', which selects the collector array into which the beams are measured (see section on the IsoPrime collector array).

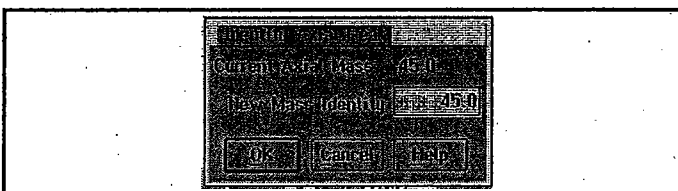
Examples of the 'Beam Mask' are given below:

If mass 45 is selected (measuring CO₂) then the following mask is used;

| | |
|------------------|-----------------|
| Beam 1 (mass 44) | Low 1 Collector |
| Beam 2 (mass 45) | Axial Collector |
| Beam 3 (mass 46) | High Collector |

If mass 29 is selected (measuring N₂) then the following mask is used;

| | |
|------------------|-----------------|
| Beam 1 (mass 28) | Low 1 Collector |
| Beam 2 (mass 29) | Axial Collector |
| Beam 3 (mass 30) | High Collector |



The 'Identify Axial Peak' dialogue box displays the 'Current Axial Mass' (also shown in the monitor window) which is the mass, the mass spectrometer accepts as the axial mass. However, if the magnet current or accelerating voltage has been moved by the user then a new axial mass must be identified in the 'New Mass Identity' entry field.

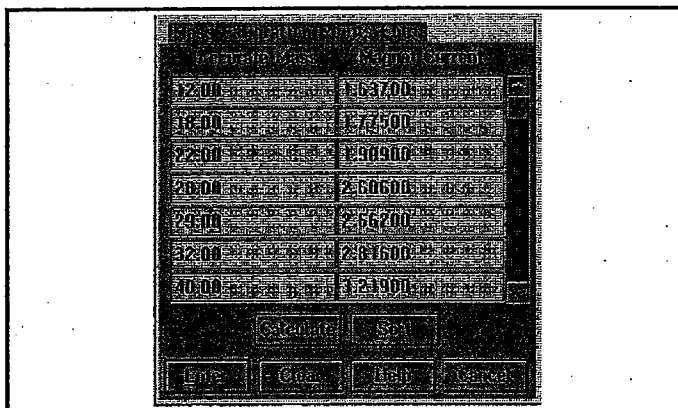
Note: Always identify the current axial mass when changing gas species.

The dialogue box has the following push buttons:

- 'OK' enters the 'New Mass Identity' given in the entry field and exits the dialogue box.
- 'Cancel' exits the dialogue box without taking any actions.
- 'Help' gives information on the operation.

Mass Calibration

This menu option opens the 'Mass Calibration Points Entry' dialogue box, which enables the mass spectrometer to be mass calibrated (i.e. the values obtained for mass value against magnet current are input into the computer in this dialogue box).



The dialogue box contains a list box with 2 entry fields on each line. The entry fields expect the 'Accurate Mass' and the 'Magnet Current' at which the mass is found.

Note: The list box has no limit to the number of peaks and the greater the number of peaks input the more accurate the mass calibration will be.

The dialogue box also has the following push buttons:

- 'Clear' removes all the entries from the list box (i.e. all the mass and magnet current data).
- 'Calculate' performs a line fit on the data points entered in the list box, which will be used to fill in the gaps in the data. This must be selected prior to entering the data.
- 'Sort' puts the entries in the list box above in mass order, with the lowest mass number at the top.
- 'Enter' saves the data to disc and exits the dialogue box.

Peak Centre

This menu option will automatically set the accelerating voltage to focus (centre) the mass in the axial collector. This is accomplished using the scan procedure file 'Centre' to scan the axial collector. If a peak is found within the scan range then the accelerating voltage is set to the peaks central value (marked with a broken vertical line). The peak shape is displayed, and may be printed out if desired, the window will stay open until closed by the operator.

Analysis

The analysis menu provides the facility for the acquisition of data through the development of different methods for each type of analyses required. A method contains all the parameters required to acquire the data, and to perform an initial data processing (AutoDP) on the acquired data. The User of the instrument defines the parameters within the Method. When a sample is analysed, the method relevant to that sample is loaded into the software. Analyses can be performed in one of two modes:

- **'Single Run'** mode for analysis of single samples
- **'Autorun'** mode for batch analysis of a number of samples.

The Analysis Menu is shown below:

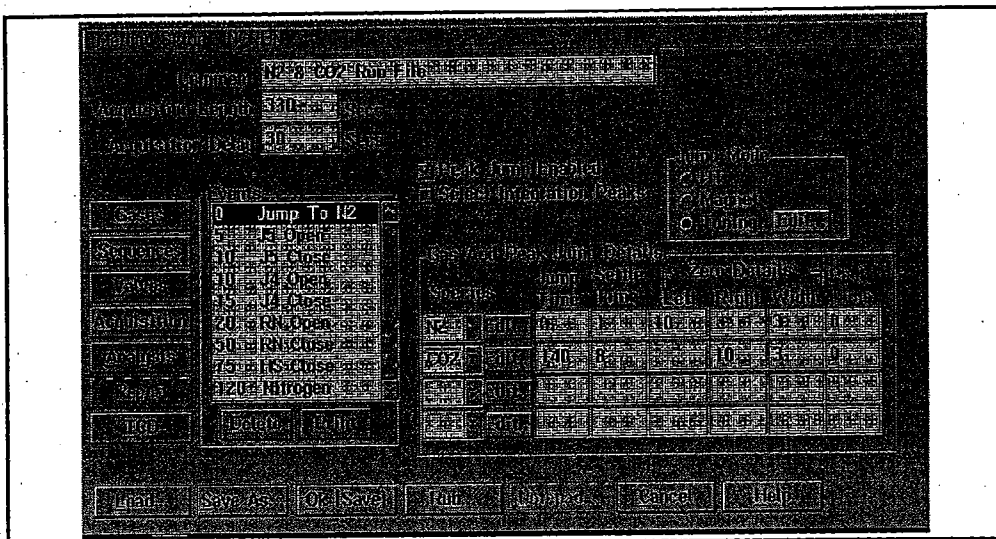


- The **'Method Setup'** option accesses the Method Setup dialogue box allowing generation and modification of methods.
- The **'Single Run'** option accesses the 'Single Run' dialogue box allowing analysis of single samples.
- The **'Edit Autorun'** option accesses a further menu in which facilities for batch analysis are available.
- The **'Autorun Stop'** facility halts any current batch analysis at the end of the current acquisition.
- The **'Parameter File Edit'** option accesses the Parameter file list, through which a series of instrument constants and settings can be accessed. The parameter files for the EA software are described later in this chapter
- The **'Batch DP'** option is used a lot in the EA software. The DP software is described in the next chapter of this manual. Worked examples for Manual data processing are also described in Chapter 6

The following sections describe each of the parameters and options encountered in the Analysis Menu. Guidance on how to use the Analysis Menu to acquire data is described in Chapter 6

Method Setup

Select 'Method Setup' option to access the 'Method Setup' dialogue box. The Method Setup dialogue box defaults to reveal the Gases overlay when the Method Setup dialogue box is accessed.



IsoPrime - EA User Manual

- The vertical set of Push buttons on the left-hand side of the dialogue box.

In this set there are 7 Push buttons:

Gases
Sequences
Valves
Acquisition
Analysis
Report
TCD

These Push buttons will call the set of parameters associated with their respective labels. For example the Push button labelled 'Gases' will call all the parameters which are associated with the information required to analyse a particular gas species (i.e. the reference gas calibration data, whether peak jumping will be required during the data acquisition, and if so, which gas to peak jump to etc.)

The set of parameters called by a particular push button will appear on the overlay area on the right hand side of the dialogue box.

- The 'Events' window.

The Events window lists all the events occurring during data acquisition using the currently loaded method. Individual Events can be highlighted to reveal the appropriate programming overlay. For example highlighting the '0 Jump To CO₂' event will reveal the Gases overlay. Individual Events can be deleted from the Events List using the 'Delete' push button. The Events List can be printed to hardcopy by clicking the 'Print' push button.

- The Jump mode and Peak jump options above the overlay area. These options allow the operator to select which gas species are to be analysed and define how the mass spectrometer automatically retunes the source to the different gas species.

- The overlay area on the right hand side of the 'Events' window.

This area is reserved for the set of parameters called by a particular push button on the left-hand side of the dialogue box. The Gas overlay appears when the 'Gases' push button is depressed, the Sequences overlay replaces it if the 'Sequences' push button is pressed in and so forth for the remaining 5 overlays.

The software remembers the last overlay used. On requesting 'Method Setup', therefore the last used overlay will appear. If none were called since the program was booted up, then the gas overlay appears by default.

- The horizontal set of Push buttons on the lower part of the dialogue box.

- 'Load' loads an existing method into the Method Setup facility. The Load Button reveals the 'Load Method File' list box. To load a method, either type in the name of the desired method in the 'Filename' entry field, or highlight the desired method name in the Filename list using the mouse cursor.
- 'Save As' creates a method under a new file name. The 'Save Method File As' dialogue box is revealed.. To generate a new method, alter an existing method to the desired configuration and save it under a new name by typing a new filename into the 'Filename' entry

IsoPrime - EA User Manual

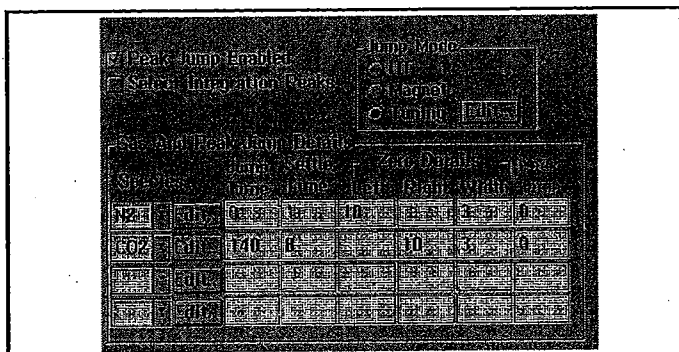
- **'OK (Save)'** saves the current settings in the Method Setup systems under the current filename.
- **'Run'** accesses the **'Single Run'** dialogue box for performing a single analysis.
- **'Notepad' ****** This is not implemented on this version of the software ****
- **'Cancel'** closes the **'Method Setup'** dialogue box without saving any alterations.
- **'Help'** accesses the help facility associated with the Method Setup dialogue box, and with the currently selected Method Setup panel.
- **'Acquisition' ****** This is not implemented on this version of the software ****
- **'Analysis'** reveals the Analysis overlay and gives access to all the analysis parameters that will be used during the automatic data processing at the end of the data acquisition cycle.
- **'Report' ****** This is not implemented on this version of the software ****
- **'TCD'** reveals the TCD panel, providing facilities for limited integration if peaks on the TCD trace.

Gases Overlay

Gases' reveals the Gases, Peak Jump and the zero details panel.

Clicking on the **'Gases'** push button, or on a **'Jump To'** entry in the Events List calls the Gases overlay.

The gases overlay is shown in the figure below. The overlay allows selection of gas species to be analysed, and specifies the points at which instrument zeros are to be measured. Note, in this example where peak jumping is enabled only one zero position for both nitrogen and carbon is selected. For nitrogen it is generally set to the left of the reference pulse and for carbon to the right of the reference pulse. A maximum of two zero positions is allowed for any gas species.

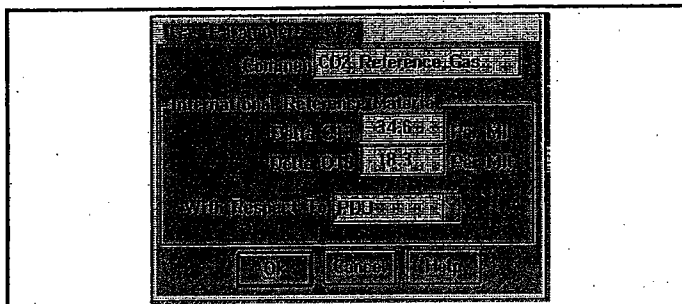


The overlay has the following features:

- **'Peak Jump Enabled'** tick box. This facility is very often used with the IsoPrime EA. The selection of this box expands the gases overlay to allow for up to 4 gas species to be analysed.

- **'Select Peaks for Integration'** tick box. This facility is not normally used with the IsoPrime-EA and the box should not be ticked.

Gas **'Species'** combo box. This facility specifies the gas species to be analysed during acquisition. Click on the right-hand downward-pointing arrow to reveal the gas species list. Double - click on the choice of gas to be analysed. The isotopic composition of the relevant reference gas is entered in the **'Gas Parameters'** dialogue box, accessed by clicking on the right-hand **'Edit'** push button. The **'Gas Parameters'** dialogue box enables entry of the details of each reference gas. The Gas Parameters dialogue box for CO₂ is shown in the figure below



This dialogue box has the following features:

- **'Comment'** Entry Field: Allows entry of up to 32 text characters forming a comment on the reference gas.
- **'Delta C13 Per Mil'** Entry Field: Contains the Craig - corrected $\delta^{13}\text{C}$ (‰ PDB) value for the reference CO₂.
- **'Delta O18 Per Mil'** Entry Field: Contains the Craig - corrected $\delta^{18}\text{O}$ (‰ PDB) value for the reference CO₂.
- **'With Respect To'** Combo Box: This facility contains a list of all the international reference standards, for example PDB, Air, CDT etc. Click on the right-hand, downward-pointing arrow to reveal the list, then double click on the choice of international standard.
- **'Ok'** push button: Saves any modifications and closes Gas Parameters dialogue box.
- **'Cancel'** push button: Closes the dialogue box without saving any changes.
- **'Help'** accesses the help facility associated with the dialogue box.
- **'Zero Details Entry Field' (Left and Right)**: Specify here the time, in seconds, after the start (left value) and before the end (right value) of acquisition at which instrument zeros will be measured. There are 10 integration points per second.
- **'Zero Details Entry Field (Width)'**: Enter here the number of seconds used for the measurement of the instrument zeros.
- **'Peak Time'**: This facility is greyed out, therefore not accessible, unless the **'Select Integration Peak'** tick box has been ticked. This facility is not normally used with the IsoPrime-EA.

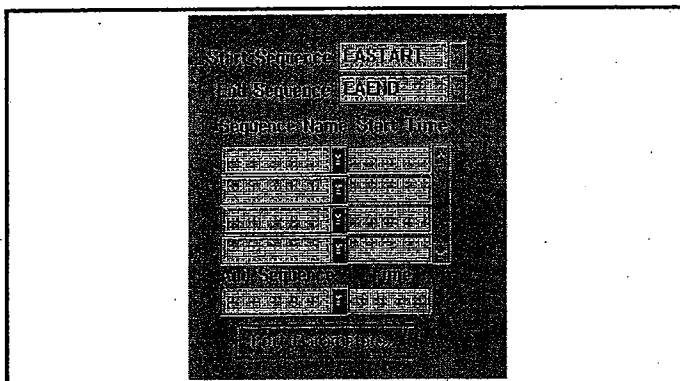
Jump Mode

The mass spectrometer can essentially jump between different masses in by three distinct methods.

- **HT:** Using the general mass spec equation of Section 3, the software recalculates the new acceleration voltage for the next mass to be analysed.
- **Magnet:** The software selects the value of the new magnet current as specified in the mass calibration table which sits in the desktop under Tests.
- **Tuning:** Selecting the activated **EDIT** button accesses the load tuning file window which allows the user to define which tuning file is reloaded when jumping to a particular species. Tick the gas option and select which tuning file to associate with it using the combo box.

Sequences Overlay

'Sequences' calls the sequence overlay containing details of start and stop sequences, and allows entry of parameters used in extra, user-defined sequences. It is shown in the figure below.



This overlay has the following features:

- **'Start Sequence' Combo Box.** Specifies the start sequence to activate the acquisition process. Click on the right-hand, downward -pointing arrow to reveal the list of available sequences. Double - click on the desired sequence for starting the acquisition procedure.
- **'End Sequence' Combo Box.** Specifies the end sequence, which terminates the acquisition process. Click on the right-hand, downward-pointing arrow to reveal the list of available sequences. Double - click on the desired sequence for ending the acquisition procedure.
- **'Sequence Name'** If the particular application requires hardware that is additional to the standard hardware supplied with the IsoPrime-EA, then this hardware may be controlled through the use of user specified sequences. The names of these sequences are then declared in these fields. The sequences can then be activated before, during or after an acquisition by declaring the Start Time of each of these additional sequences in the adjacent field set.

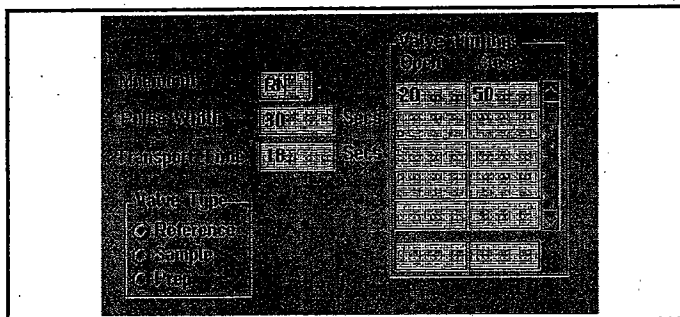
IsoPrime - EA User Manual

- **'Start Time'** The Start time of any additional user defined sequence is declared in this list, adjacent to the name of the corresponding sequence.
- **'Add Sequence'** Combo Box. Additional sequences are added to the existing list, using this entry field.
- **'Time'** The time for any additional sequence, to be added to the existing list is declared in this field.

Valves Overlay

'Valves' calls the Valves overlay allowing programming of all the valve actions required during an acquisition

Clicking on the **'Valves'** push button, or on a valve action in the Events List, accesses the Valves overlay. The timing of all the valve actions in a method are controlled through the Valve Panel.



This panel has the following features:

- **'Mnemonic'** Entry Field. Contains the mnemonic for any valve for which timed actions are to be entered. The standard software recognises RG for the CO₂ Reference Gas Valve, RN for the N₂ Reference gas valve, and HS for the Heart Split Valve. Enter the appropriate mnemonic to edit the desired valve timings, then press return.

Note that the HS valve defined as a sample valve does not control an actual valve in this application. It is used solely for the purpose of defining a window within which the program will search for sample peaks to integrate. The user **must** ensure that any sample peaks to be integrated are **wholly** contained within this window. Particular care must be taken when defining the right limit for the integration window to ensure that any peak tailing, if present, is included within the integration window. Integration stops at the end of the integration window, it does not stop at the end of a peak, unless this is contained within the integration window.

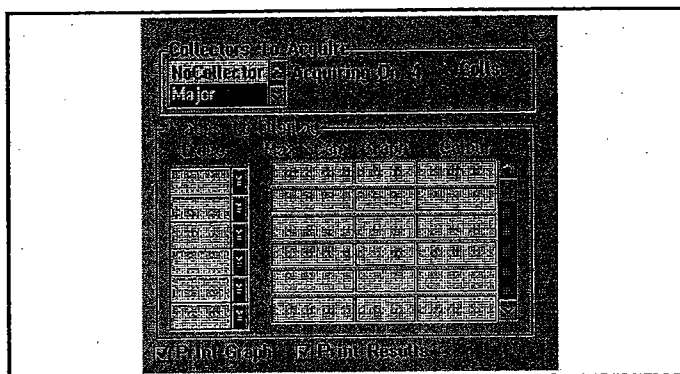
- **'Pulse Width'** Entry Field. Contains the width, in seconds, of any reference gas pulse. This entry field remains greyed out when a mnemonic for a non-reference gas valve is entered in the Mnemonic Entry Field.
- **'Transport Time'** Entry Field. This contains the time delay, in seconds, between the valve action and the effect observed in the mass spectrometer. For example the time delay between opening the RG valve and the reference gas beams appearing in the collectors.

IsoPrime - EA User Manual

- **'Valve Type' Radio Buttons.** Specifies which type of valve (Reference, Sample or Prep) for each mnemonic. Thus the HS valve is specified as a Sample valve, RG as a reference valve. Valves incorporated into custom-built preparation systems can be entered as Prep valves.
- **'Valve Timings' Window.** The Valve Timings Window lists the action times for the valve mnemonic in the Mnemonic Entry Field. To access the Valve timings for a particular valve enter the desired mnemonic in the Mnemonic Entry Field, or double click on a suitable valve action in the Events list.
 - **'Valve Timings Open'** lists the time(s) in seconds at which the specified valve will be open. The vertical scroll bar can be used to view the entire list.
 - **'Valve Timings Close'** lists the time(s) in seconds at which the specified valve will be closed. For reference valve timings this will be automatically calculated by adding the value in the Pulse Width Entry Field to the corresponding Valve Open time. For Sample and Prep valve times the operator must determine the closure times. The vertical scroll bar can be used to view the entire list.

Acquisition Overlay

Clicking on the 'Acquisition' push button calls the Acquisition overlay. The Acquisition Panel provides the facility to customise the data acquisition window and the final default print report.



This panel has the following features:

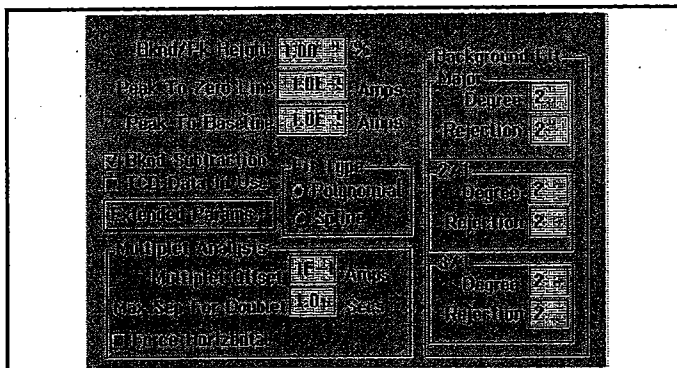
- **'Collectors to Acquire'** combo box is used to select the collectors and detectors used for the acquisition. In the EA software, for CO₂ and N₂ analyses, this will normally include Major, Minor1, Minor2 and TCD
- **'Traces to Display'** panel. **** This facility is greyed out and is not accessible ****
- **'Print Graph'** Tick Box. If the user requires to print the Mass Spectrometer trace acquired during an acquisition, on the final default report, then a tick should be entered in this field.

- **'Print Results' Tick Box.** If the user requires to print the results of an analysis on the default run report, then a tick should be entered in this field.

This facility is particularly useful when performing a Batch analysis. If the field is left unticked, then the results from each single analysis within the batch will not be printed at the end of each analysis but will be delayed until the batch summary report is printed. The final batch report has a much more condensed format thus avoiding large quantities of printer paper being used in the event that all intermediate results are not required.

Analysis overlay

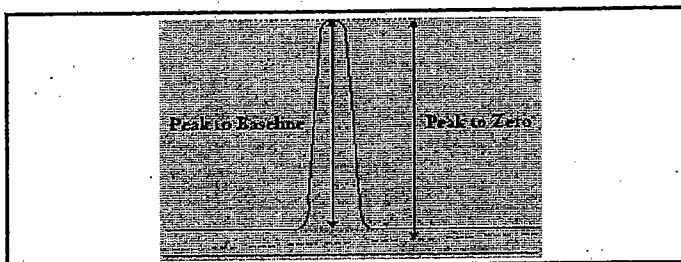
The Analysis overlay is called by clicking on the Analysis push button. The parameters in the Analysis Panel control the protocols for the automatic data processing (AutoDP), occurring directly after the data acquisition process. The parameters can be modified in the manual data processing (DP) software. The parameters are divided between two windows. The secondary, or extended parameters, are called by clicking on the Extended Parameters button. The Extended Parameters are described in a later section.



This panel has the following features:

There are two parameters that control the size limits for peak integration during data processing. Below these limits peaks will not be integrated, the limits are set in the entry fields below:

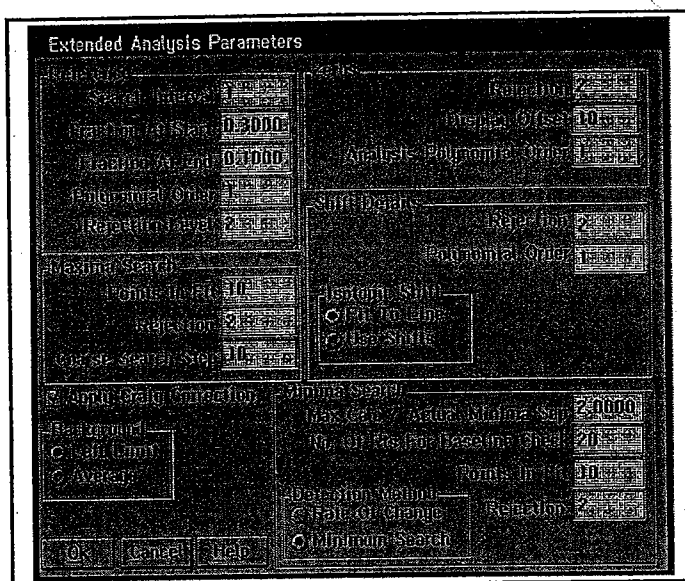
- **'Peak to Zero Line' Entry Field.** The value entered here (in Amps) represents the height threshold, measured from the Major zero line to the peak apex, below which peaks are not integrated.
- **'Peak to Baseline' Entry Field.** The value entered here (in Amps) represents the height threshold, measured from peak baseline to apex, below which peaks are not integrated.
- **'Bknd/Pk Height'.** The value entered here represents the height of the background at the left limit of a sample peak, expressed as a percentage of the sample peak height. Peaks, for which this percentage is below that defined, will not be background subtracted. They will however be zero subtracted. Peaks for which this percentage is greater than that defined, will be background subtracted in addition to the zero subtraction.



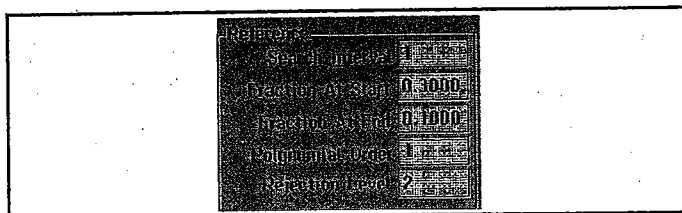
This feature allows background subtraction to be performed on an entire mass chromatogram, yet selecting peaks automatically along this chromatogram which are very large compared to the background and for which background subtraction is not necessary.

- **'Multiplet Analysis'** Parameters control the protocols for recognising doublets and multiplets occurring on complex chromatograms. Where a multiplet is recognised, a different method of peak integration is employed.
 - **'Multiplet Offset' (Amps)** Entry Field. This provides a facility for identifying those doublets, or multiplets containing significantly overlapping peaks. The value entered here (in Amps) represents a height threshold, measured from the multiplet baseline to doublet minima, above which the peaks will be integrated using the doublet method.
 - **'Maximum Separation for Doublet' (seconds)** Entry Field. This parameter helps identify those peaks that are sufficiently close to be considered a doublet or multiplet series. The value entered here (in seconds) represents the interval between peaks within which the peak pair is classified as a doublet, and integrated accordingly. The interval is measured from one peak right limit to the next peak left limit.
 - **'Force Horizontal'** tick box. When selected the multiplet baseline is drawn as horizontal, rather than as a sloping fit between the left and right limits for this multiplet. The lowest point is used to draw this line.
- **'Background Subtraction'** parameters influence the protocols for controlling the background correction algorithms.
 - **'Bknd Subtraction'** tick box activates the background subtraction process during AutoDP.
 - **'Fit Type'** radio button selected specifies the desired type of line, either polynomial curve or spline function, for the background correction protocols. The spline function is not yet available.
 - **Major Degree and Rejection: ****** This facility is greyed out and is not accessible ****
 - **2/1 Degree and Rejection:** the order of the polynomial curve used for modelling the 2/1 ratio trace for background subtraction is entered in the Degree entry field. The curve is drawn through the background points on the 2/1 ratio trace. Those points falling more than the number of standard deviations specified in the Rejection entry field from the line will be disabled. Disabled points will be marked in red on the graphics.

Clicking on the **Extended Parameters** button in the **Analysis Overlay** calls the **Extended Analysis Parameters** dialogue box.



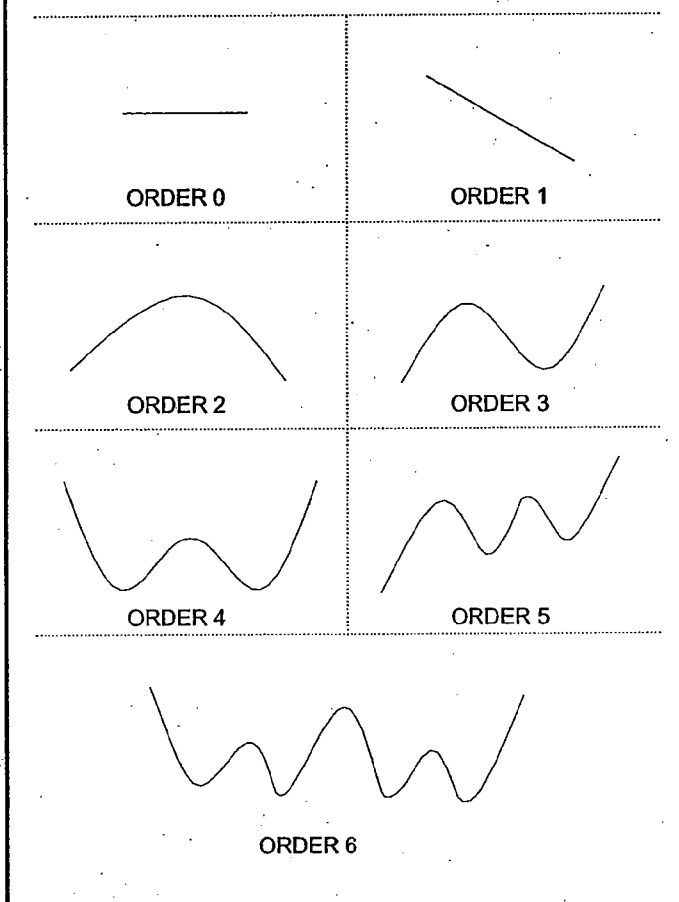
- **'Reference' Parameters** control the protocols or the search and integration of reference peaks.



- Page 53

- 'Fraction at Start' entry field is the portion at the start of the reference peak which is disregarded during integration of the reference peak
- 'Fraction at End' entry field is the portion at the end of the reference peak which is disregarded during integration of the reference peak.
- 'Polynomial' entry field. The order of the curve drawn through the reference ratio points (both 2/1 and 3/1 ratios) is specified in this entry field. It is recommended that order 1 is always used.

A polynomial defines a mathematical curve. The number of turning points for this curve is defined by the order of the polynomial. Examples of general shapes for orders 0 to 6 are given below



- 'Rejection Level' entry field specifies the rejection level, in standard deviations, outside which individual reference peaks are not used. Disabled peaks are coloured red.
- 'Maxima Search' parameters influence the protocols used for finding the maxima of each integrated sample peak.
 - 'Points in Fit' entry field specifies the number of readings used in drawing the polynomial curve for determining the peak maxima.
 - 'Rejection' entry field specifies the rejection level, in standard deviations, outside which individual maxima-search points are not used.

- **'Coarse Search Step'** entry field specifies the interval (i.e. the number of scans) used in the coarse-search step of finding the sample peaks. Note that the scanning rate is 10 per second, therefore an entry of 10 would result in a coarse search step of 1 second along the chromatogram.
- **'Minima Search'** analysis parameters influence the protocols for accurately finding the left and right limits of each sample peak. The protocol of determining whether a sloping or horizontal baseline fit should be used is also controlled here.
 - **'Detection Method'** radio buttons specify which method is used for detecting the peak minima:
 - **'Rate of change'**. **** This facility is greyed out and is not accessible ****
 - **'Minimum Search'**. The peak limit is detected at the lowest measured reading around the peak maximum.
 - **'Max Calc/Actual Minima Sep'** entry field specifies the limit, in seconds, between the measured peak minimum and the minimum calculated using the polynomial curve method. If the difference is within the specified limit, then the polynomial minimum is used, otherwise the measured minima is used.
 - **'No of Points for Baseline Check'** entry field specifies the number of scans to the left of the peak minima which are analysed to determine whether the peak baseline cuts the Major Beam trace. If the major is cut, then a horizontal baseline fit is used.
 - **'Points in Fit'** entry field specifies the number of points taken for second order polynomial fit to find the minimum i.e. start at integration limit.
 - **'Rejection'** entry field specifies the rejection level, in standard deviations, outside which individual minima-search points are disabled.
- **'Zeros'** parameters control the drawing of the zero subtraction line, and the performance of the display zero. The Positions of the zero lines are specified in the Gases Panel.
 - **'Rejection'** entry field specifies the rejection level, in standard deviations, outside which individual zero points are disabled.
 - **'Display Offset'** entry field specifies the position of the zero measurements, in scans after the start of acquisition, taken in order to plot accurately the ratio traces during data acquisition.
 - **'Analysis Polynomial Order'** entry field specifies the order of the polynomial curve drawn through the zero analysis points. A first order polynomial should always be used.
- **'Shift Details'** parameters allow selection of the method for compensating for the Isotopic Shift between the heavy and light isotope - rich species:
 - **'Isotopic Shift'** radio buttons:
 - **'Use Shifts'** Radio Button: the isotopic shift values of the individual peaks are used to calculate the δ values of the particular sample peaks.

IsoPrime - EA User Manual

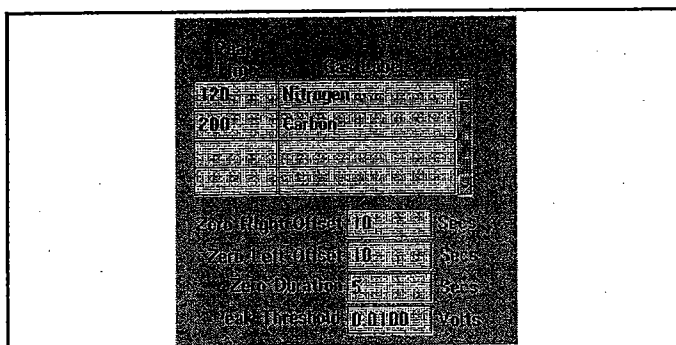
- **'Fit to Line' Radio Button:** the individual Isotopic Shift values are plotted as a function of time, and a best fit polynomial drawn through them. The values used in the isotopic calculations are then extrapolated from the best fit line.
- **'Rejection' entry field** specifies the rejection level, in standard deviations, outside which individual isotopic shift values are disabled during calculation of the Isotopic Shift line.
- **'Polynomial Order' entry field** specifies the order of the polynomial curve used in the calculation of the Isotopic Shift line. A first order polynomial should always be used.
- **'Apply Craig Correction' tick box** enables the application of the Craig correction during $\delta^{13}\text{C}$ (and $\delta^{18}\text{O}$) calculation.
- **'Background' radio buttons:**
 - **'Left Limit' Radio Button:** specifies that the automatic background points selected during AutoDP are positioned at the left limits of the integrated sample peaks
 - **'Average' Radio Button:** specifies that the automatic background points selected during AutoDP are an average of the left and right peak limits of the integrated sample peaks, and positioned at the left limits of the sample peak.
- **'OK' push button** saves any alterations and closes the Extended Analysis Parameters dialogue box.
- **'Cancel' push button** closes the Extended Analysis Parameters dialogue box without saving any changes
- **'Help' push button** accesses the help facility associated with the Extended Analysis Parameters.

Report Overlay

**** This facility is greyed out and is not accessible ****

TCD Overlay

This facility provides the instructions required for the integration of sample peaks acquired by the TCD



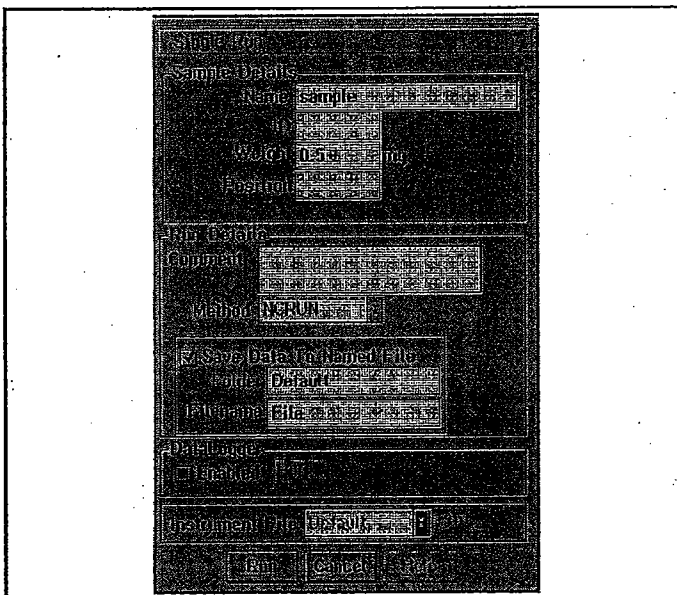
The panel has the following features:

- **'Peak Time'** entry field list specifies the approximate retention times, in seconds, of those peaks to be integrated.
- **'Species Label'** entry field list enter the desired label for each peak to appear on the results printout.
- **'Zero Right Offset'** entry field enter the time, in seconds prior to the end of acquisition, at which the right TCD zero measurement will be taken
- **'Zero Left Offset'** entry field enter the delay, in seconds after the start of acquisition, after which the left TCD zero measurement will be taken.
- **'Zero Duration'** entry field enter the time period, in seconds, over which the left and right TCD zeros will be measured.
- **'Peak Threshold'** entry field enter the peak height threshold value, in volts, below which the specified TCD peak will not be integrated.

Analysis

Single Run

The Single Run Option provides the facility for the collection of data from individually performed sample analyses. Selecting this menu option opens the 'Single Run' dialogue box.



This dialogue box has the following features:

- **'Sample Details' panel**
The details of the sample are entered into the appropriate entry field.
- **'Name'** entry field provides 32 characters for recording the full name of the sample. This name is not used for data storage, but appears on the results printout.

IsoPrime - EA User Manual

- **'ID'** entry field provides 32 characters for further identification of the sample, for example the number of a repeat analysis. The ID entry is printed on the results printout.
- **'Injection Size' (mg)** entry field enters the amount of sample to be injected into the elemental analyser. This is used for the calculations of the elemental composition of the sample. An accurate entry of the sample weight must therefore be made for the final calculation to yield an accurate evaluation of the sample elemental composition.
- **'Position'** entry field specifies the position of the sample on the sample carousel. This may be left blank as the entry is used solely for information purpose.
- **'Run Details' panel**

This panel has the following features:
- **'Comment'** entry field provides 200 characters of space for recording specific information about the Single Acquisition, for example EA conditions etc.
- **'Method'** combo box facilitates selection of the method to be used for the Single Acquisition. Click on the right-hand, downward pointing arrow to reveal the list of available methods.
- **'Save Data to Named File'** tick box when selected, the data and AutoDP parameter under a file name and folder specified in the Folder and Filename Entry Fields. When the tick box is not selected, the data is saved under a TEMP filename in a TEMP folder.
- **'Folder'** entry field provides 8 characters to specify the name of the Folder in which the data file will be stored.
- **'Filename'** entry field provides 5 characters for naming the file in which the data will be stored.
- **'Data Logger' panel**

This facility allows transfer of data into spreadsheets.
- **'Enabled'** tick box when activated the appropriate AutoDP data will be stored in a text file (.txt).
- **'Edit'** push button accesses the Data Logger dialogue box.
- **'Run' push button**

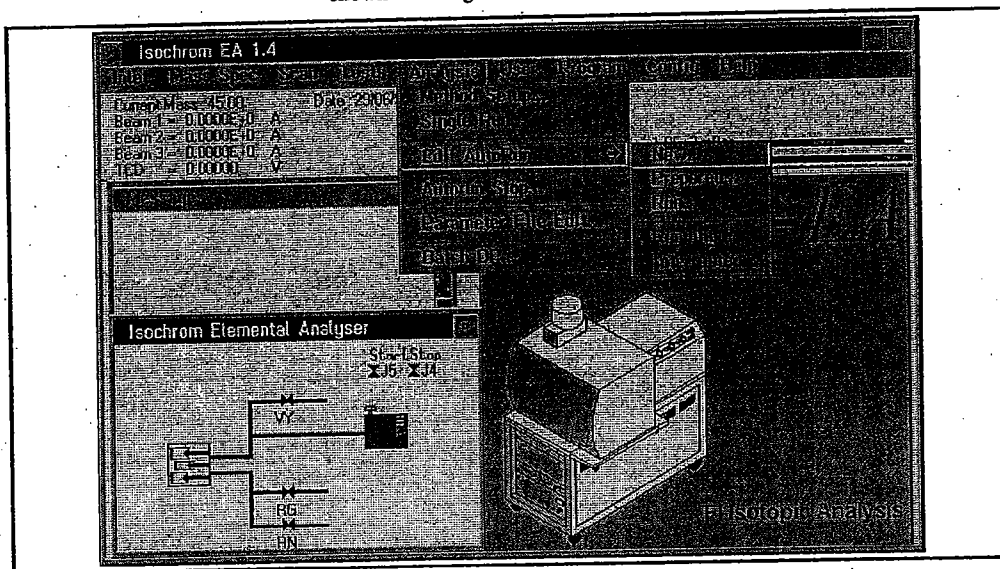
Clicking on the run button initiates the data acquisition process, and closes the Single Run dialogue box.
- **'Cancel' push button**

Closes the Single Run dialogue box without initiating the data acquisition process.
- **'Help' push button**

Accesses the help facility associated with the Single Run Dialogue Box.

Edit Autorun →

The 'Edit Autorun' option accesses the Edit AutoRun cascade menu that provides the facility for the setup and execution of batch analyses. Batch analyses are performed in 'AutoRuns'. The cascade menu is accessed by clicking on the right-pointing arrow in the Edit Autorun option, and is shown in the figure below.

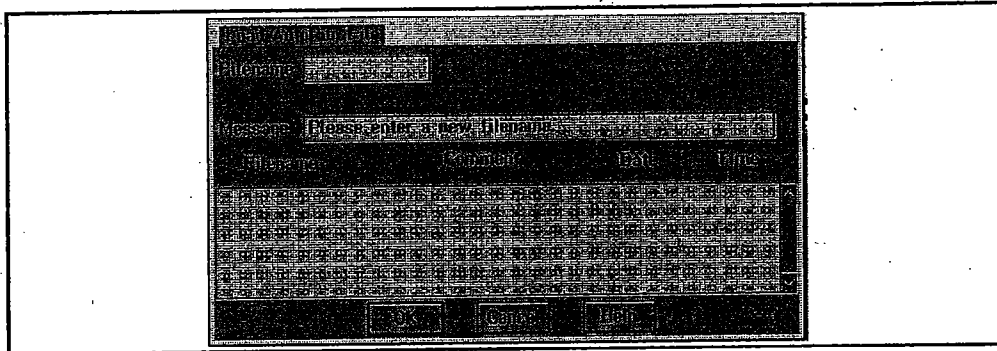


This menu option allows for the following sub-menus to be selected by the sub-menu arrow:

- **'New'** option allows setting up of new autoruns via the Autorun Batch Dialogue box.
- **'Prepared'** option allows access to details of AutoRuns that have been prepared, but have not been executed.
- **'Run'** option allows review of the details of autoruns that have been executed.
- **'Running'** option accesses details of an autorun currently being performed. When no autorun is being performed this option is inactive and appears 'greyed out'.
- **'Interrupted'** option accesses details of any autoruns that have been interrupted (temporarily halted). When no autorun has been interrupted this option is inactive and appears 'greyed out'.

'Load AutoRun File' dialog box

The details of each autorun are stored as an autorun file. When an option in the Edit Autorun cascade menu is selected, the Load AutoRun File dialogue box appears.

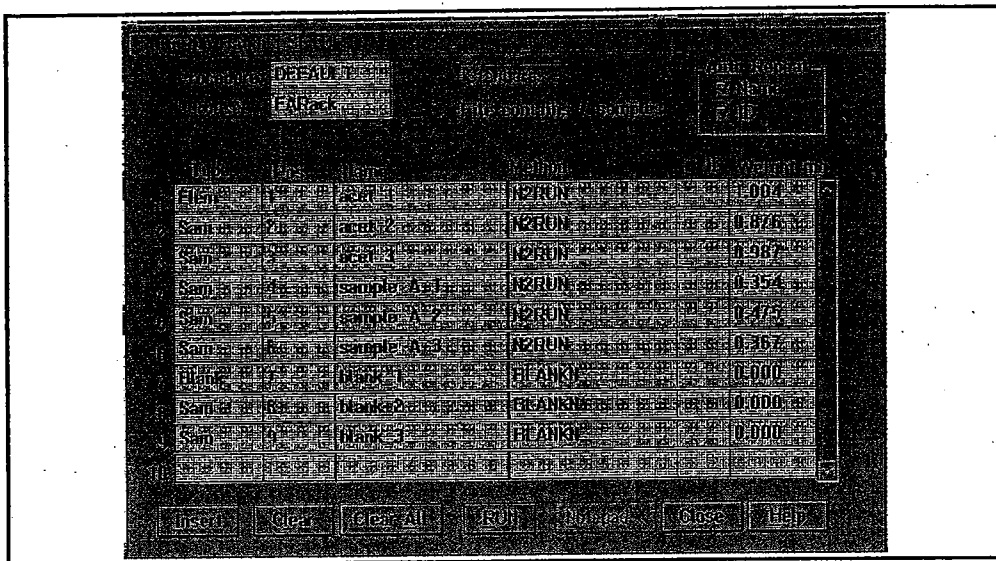


This dialogue box display has the following possibilities:

- When 'New' is selected, a list of all autorun titles is displayed. A 'Please enter a new filename' message prompts the user to enter a filename which has not been used previously. By examining the list displayed, it is possible to check the names that have been used before. If a name that has been used previously is typed in, then a message appears, prompting a different filename to be chosen. If the same filename needs to be used repeatedly, then it must be followed by a number, which can be incremented, such that the overall filenames are all different. The software will never accept two identical names in order to guarantee that acquired data files can never be overwritten.
- When 'Prepared' is selected, the autoruns that have been prepared, but not yet run, are listed. The message in this case prompts the user to select a name from the list displayed.
- If the 'Run' option is chosen, those autoruns that have been executed will be listed. The message prompts the user to select a name from the list displayed.

Autorun Batch Dialog Box

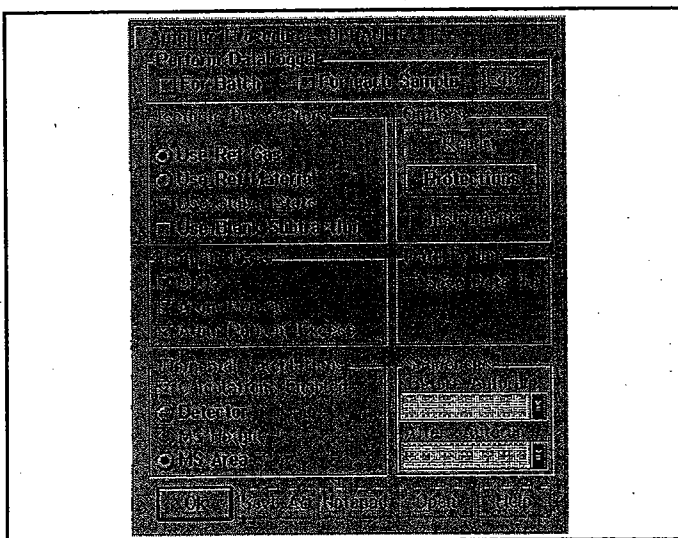
AutoRuns are setup in the 'Autorun Batch' Dialogue Box. The dialogue box can be accessed through the New, Prepared, Run, Running and Interrupted options of the 'Edit Autorun' sub-menu (cascade menu).



The dialogue box has the following features:

- 'Procedure' entry field accesses the AutoRun Procedure dialogue box. This provides the facility to set up the detailed procedures for the execution of AutoRuns.

In order to edit the procedure:

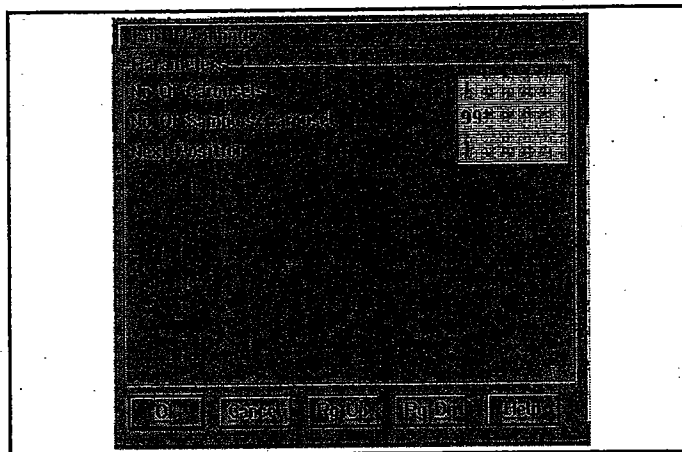


Click once on the name of the procedure entered in the 'Procedure field'.

This action reveals the 'Edit' push button. Click on the 'Edit button' once, this reveals the 'Procedure window'.

The procedure for the batch analysis of samples is set via this window. It enables the selection of a number of features required for the calculations and the final batch output.

- **Data Logger facilities** are enabled by inserting one tick in the tick boxes 'For Batch' and 'For each Sample'. It is possible to select none, either option or both options simultaneously.
When the 'For each Sample' option is selected, the 'Edit' push button appears to provide a series of windows which define the parameters which the user wishes to download to the data logger and the name of file and folder where this information needs to be stored.
When the 'For Batch' option is selected, then only the default template for this file can be used. That is, there is no optional template for batch results.
- The **'Isotopic Calculations'** can be performed either by using the reference gas or an internal standard inserted within the batch. Select the required option by pressing one of the two radio buttons.
Furthermore, if the subtraction for the potential presence of blanks is to be included, then the **'Use Blank Subtraction'** tick box needs to be ticked.
The use of **'Global Statistics'** is not available in this version of software. The facility is therefore greyed out and is not accessible.
- The **'Isotopic data'** can be reported in any of the three units specified or in any combination of these. Make appropriate selections by placing ticks in the appropriate tick boxes.
- The **'Elemental Calculations'** may be performed (if required), either by using the output signal from the TCD detector or using the output from the mass spectrometer signal. For the latter, in this version of software, the calculations are made using peak areas. In later versions of this software, it will also be possible to perform this calculation using the peak heights as acquired by the mass spectrometer. The preferred mode of calculation is selected by the choice of radio button.
- All other features shown on this window are not available in this version of software. They are greyed out and therefore not accessible.
- **'Carousel'** This file defines the characteristics of the carousel used with the elemental analyser. By clicking on the Carousel name entry field, the 'Edit' button appears. One click on the 'Edit' button reveals the carousel parameters window.



- **'Standards'** In this application, the 'standards' push button is greyed out. The use of standards is not applicable when the prep system in use is an elemental analyser.
- **'Auto Repeat'** tick boxes have the following features:
 - **'Name'**: This facility is used when entering sample details into the AutoRun Batch dialogue box. When activated, automatically enters the previously used sample Name in the sample Name entry field. If the Sample name is suffixed by a number, e.g. Sample1, the number will be automatically incremented by 1.
 - **'ID'**: This facility is used when entering sample details into the AutoRun Batch dialogue box. When activated, automatically enters the previously used sample ID in the sample ID entry field.
- **The table for Sample entries**
 - **'Type'** combo box list allows classification of the material to be analysed into Blank, Elemental and Isotopic (E & I) reference, Elemental (Elem) reference, Isotopic (Iso) reference, and Sample (Sam). Click on the combo box to make it active, then click on the right - hand, downward or upward pointing arrow to reveal the full list of options and to select one of these options.
 - **'Sam'** (sample): defines the material to be analysed as a sample material
 - **'Iso'** (isotopic reference): defines the material to be analysed as an isotopic reference, against which the isotopic compositions of samples may be calculated if so requested.
 - **'Elem'** (Elemental Reference) defines the material to be analysed as a reference material for the calculation of elemental composition of samples if this calculation is requested.
 - **'E & I'** (Elemental and Isotopic Reference) defines the material to be analysed as both an isotopic and an elemental reference, against which calculations for a sample may be performed.

IsoPrime - EA User Manual

- **'Blank'** defines the material to be analysed as a blank. A blank consists of a normal analysis cycle but in the total absence of sample material. It provides information about the signals obtained during a normal cycle, this contribution, if significant, can then be accounted for when a sample is analysed.
- **'Position'** entry field list defines the position of the sample on the carousel. It is used for information purpose only. Because the operation of the carousel is sequential only, then it is not possible to make use of the entry in this field to direct the analysis to samples other than the sample that the carousel presents to the system for analysis. The position numbers need not be entered, as they will automatically be incremented by one, each time a new line is filled.
- **'Name'** entry field list provides 32 character spaces for entering the full sample name, which is printed on the data printout. Sample names can be auto-repeated and auto-incremented.
- **'Method'** combo box list provides the facility for the selection of the appropriate method for the specified sample. Click on the entry field to activate the combo box, and use the right - hand, downward - pointing arrow to view the available methods. Click on the Edit button to access the Method Setup dialogue box.
- **'ID'** entry field list **** This facility is greyed out and is not accessible ****
- **'Weight' (mg)** entry field list specifies the weight of the material to be analysed. This needs to be entered accurately as it is used in the calculation for the elemental compositions of samples.
- **'Insert'** inserts a blank line into the autorun order for addition of samples
- **'Clear'** clears the entries along the line where the cursor is positioned.
- **'Clear All'** clears the entire autorun entries. A message will appear to prompt you to confirm that you really want to carry out this action before any clearing is actually done.
- **'Run'** initiates or re-initiates an AutoRun.
- **'Notepad'** **** This facility is greyed out and is not accessible ****
- **'Close'** closes the AutoRun Batch dialogue box. The entries are saved under the name given to this batch in the prepared autorun list.
- **'Help'** accesses the help facility associated with the AutoRun Batch dialogue box.

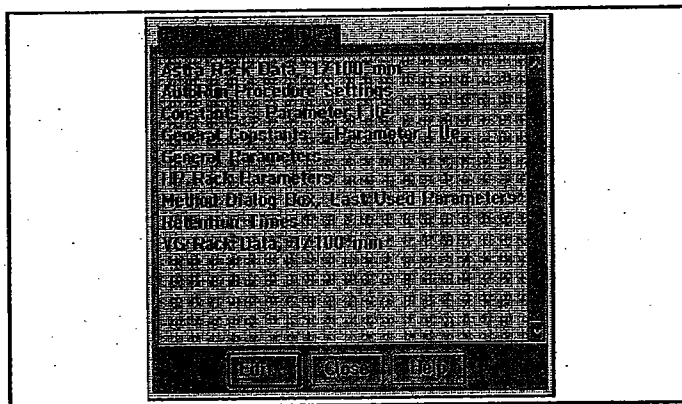
Autorun Stop

Interrupts the AutoRun in progress.

Parameter File Edit

Note: For details of the parameters see later sections of this manual.

This menu option opens the 'Parameter Files Edit' dialogue box, which allows the parameters files to be edited.



This dialogue box contains a list box containing all the parameter file names available to the user (see below), from which a file to be edited can be chosen. The dialogue box also has the following push buttons:

- 'Edit' opens the 'Edit Parameters' dialogue box, which allows you to edit any of the parameters within that file.

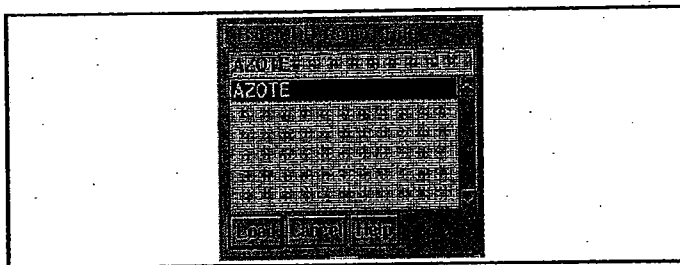
Each file contains one or more pages of parameters contained in entry fields, which can be changed as with any entry field. Please see the next section of this manual for details of the parameters.

- 'Cancel' exits the dialogue box.
- 'Help' gives information on the operation.

The parameter files available are:

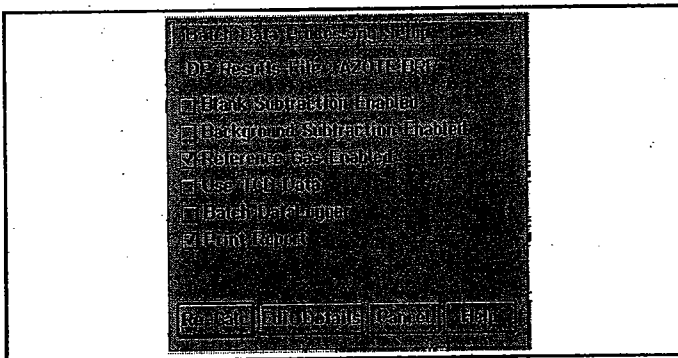
- 'Autorun Procedure Settings' accesses the threshold values for autorun protection parameters.
- 'Constants - Parameter File' accesses adjustable System and Instrument Constants.
- 'Current Rack Parameters' is not used in the EA software.
- 'General Constants' accesses fixed Instrument constants.
- 'General Parameters' accesses variable parameters associated with continuous - flow operation.
- 'HP Rack Parameters' accesses variable parameters associated with the operation of the HP7673 autoinjector.
- 'Method Dialogue Box, Last Used Parameters' accesses the set of parameters in the method that was last used.
- 'Retention Times' accesses the current values of the Retention Times universal parameters.

Batch DP Option



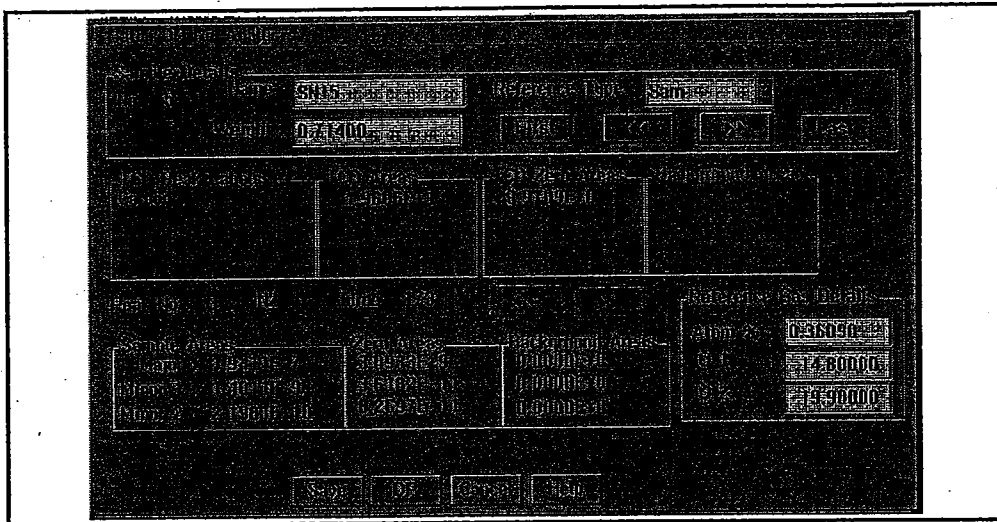
The batch DP option gives access to previously acquired data for further manual manipulation of the data prior to re-integration. Selecting this option, calls the Batch DP Report File as shown below:

The window is a full list of all the batches acquired to date and for which the data is stored on the hard disk. Any of these batches can be re-called by selecting it from this window and pressing the 'LOAD' push button. The data for this batch is loaded and the Batch Data Processing Setup window appears:



Pressing the 'Re-Calc' push button will start the re-integration process of the full batch using the instructions as defined on the Batch Data Processing Setup window. Recalculations on the full batch can be performed as many times as is required, by enabling any combination of the features offered on this window.

The 'Edit Details' push button gives access to information for each individual sample. A first window gives information on intermediate calculations; this information is non-editable and is shown in dark blue. The details, which may be altered, feature in white entry fields and include the sample name and its weight, as well as the calibration data for the reference gas used. The Batch DP Re-Analysis window is shown below:



This window contains a set of options that can be selected and deselected by using the tick boxes provided for this purpose.

From this window, it is possible to call the full manual Data Processing facilities in the software suite. These facilities are accessed by clicking on the 'DP' push button.

A full description of the manual Data Processing facilities is given in a later section of this manual.

User

The IsoPrime - EA software incorporates User levels, passwords, and a Login facility. This enables the user to protect the machine against unauthorised use, and perhaps more importantly, restrict the access of inexperienced users to portions of the software, so that the consequences of inadvertent mistakes can be avoided.

The portions of the software appropriate to the user level are:

Level 1

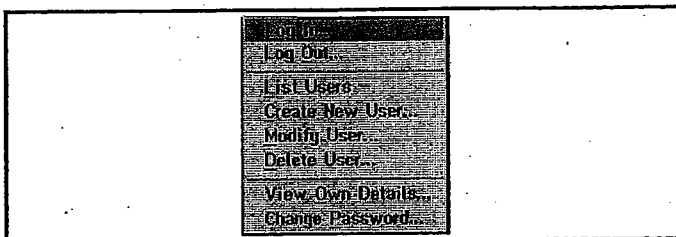
This allows access to sample batch entry, autorun start, and a restricted range of the manual control features.

Level 2

This intermediate level allows access to most of the manual control features of the instrument, and allows editing of selected run parameters etc.

Supervisor

Users at this level have access to all of the software.

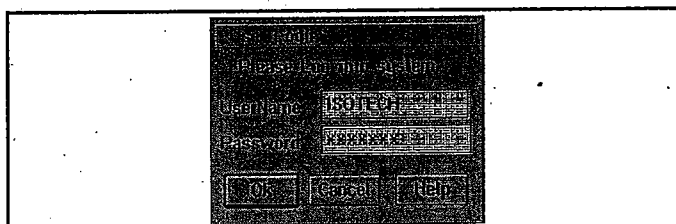


Note: When there is no user logged in, all menus except USER and HELP are disabled.

The menu options are:

Log In

This menu option opens the 'User Login' dialogue box that allows the user to log into the system.



The dialogue box has 2 entry fields that allow the user to input their 'Username' and their 'Password'.

The dialogue box has the following push buttons:

- 'OK' checks the 'Username' and 'Password' for validity, and either exits the dialogue box if they are valid giving access to the functions of the appropriate user level or opening a dialogue box giving the reason for denied access.
- 'Cancel' exits the dialogue box without taking any actions.
- 'Help' gives information on the operation.

Log Out

This menu option when selected logs the user off the system.

Notes:

- a) Only the 'User' and 'Help' menu options are now available until the user logs in again.
- b) Even with no user logged in, the software remains running. This means that you can start an Autorun, and then logout to prevent unauthorised interruptions or access.

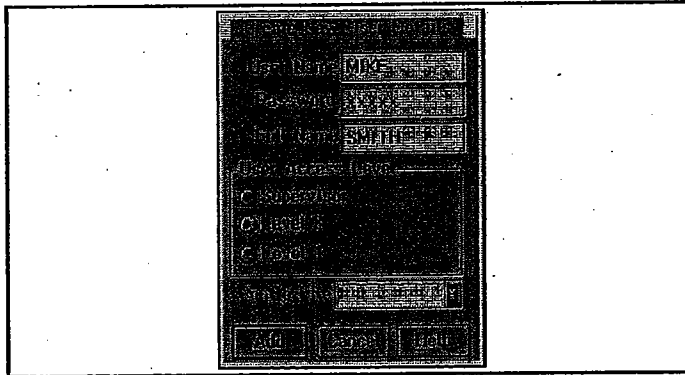
List User

This menu option opens the 'User List' dialogue box that gives a list of all authorised users.

Only Supervisor level users have access to the next group of commands in the 'User' Menu:

Create New User

This menu option opens the 'Create New User Details' dialogue box, which allows new users to be given access to the system.



This dialogue box expects the following information in the entry fields:

- **'Username'** requires the new name that the user will use to gain access to the software (up to a limit of 10 characters).
- **'Password'** requires a character set with a minimum of 5 characters known only to the new user (maximum 10 characters).
- **'Full Name'** requires the full name of the user.

The user level is then selected using the radio buttons in the 'User Access Level' section

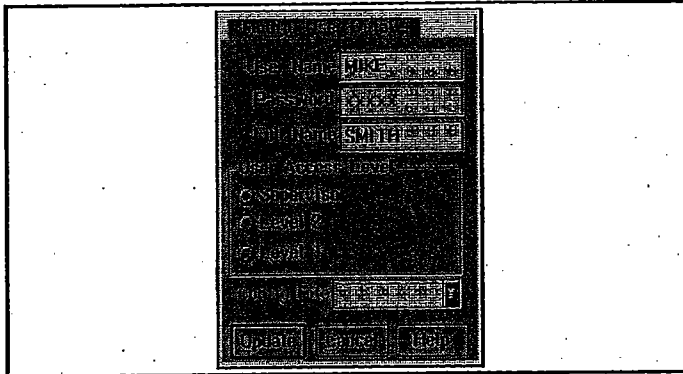
Note: The 'Config File' combo box. **** This facility is greyed out and is not accessible ****

The dialogue box then has the following push buttons:

- **'Add'** updates the user list with the new user and clears the dialogue box, allowing another user to be added.
- **'Cancel'** exits the dialogue box without taking any actions and should be used to exit the dialogue box when no further users are to be added.
- **'Help'** gives information on the operation.

Modify User

This menu option opens the 'Modify User Details' dialogue box, which allows the user access level or password to be altered.



This dialogue box expects the following information in the entry fields:

- 'Username' requires the name of the user whose details are to be modified, this once entered will load the 'Full Name' of the user, when the TAB key is used to move to the next entry field.
- 'Password' requires the new password if the password is to be modified.
- 'Full Name' gives the full name of the user once the 'Username' has been inputted.

The new user level can then be selected if required, using the radio buttons in the 'User Access Level' section

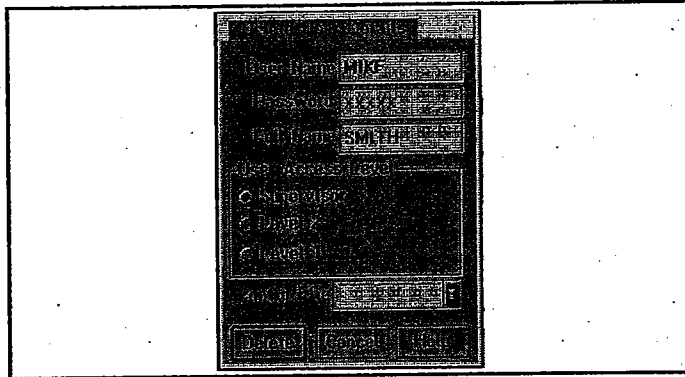
Note: The 'Config File' combo box. **** This facility is greyed out and is not accessible ****

The dialogue box then has the following push buttons:

- 'Update' changes the user access level or the password.
- 'Cancel' exits the dialogue box without taking any actions and should be used to exit the dialogue box when no further users are to be modified.
- 'Help' gives information on the operation.

Delete User

This menu option opens the 'Delete User Details' dialogue box, which allows user access to be deleted.



This dialogue box expects the following information in the entry fields:

- 'Username' requires the 'Username' whose details are to be deleted, this once entered will load the 'Full Name' of the user, when the TAB key is used to move to the next entry field.
- 'Full Name' gives the full name of the user once the 'Username' has been inputted.

The new user level is then selected using the radio buttons in the 'User Access Level' section

Note: The 'Config File' combo box. **** This facility is greyed out and is not accessible ****

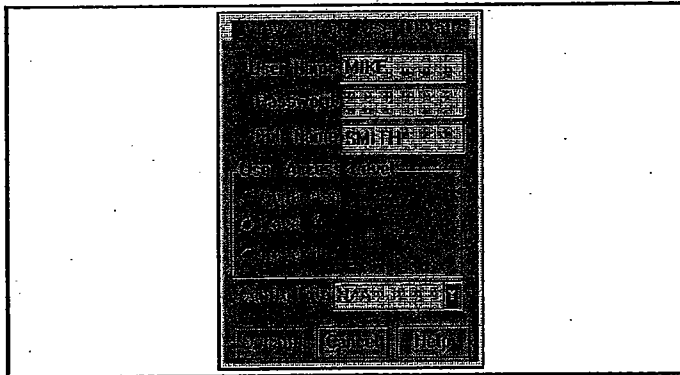
The dialogue box then has the following push buttons:

- 'Delete' opens a delete confirmation box, expecting 'Yes' (to delete the user) or 'No' (to not delete the user).
- 'Cancel' exits the dialogue box without taking any actions and should be used to exit the dialogue box when no further users are to be deleted.
- 'Help' gives information on the operation.

All users have access to the final two User menu commands.

View Own Details

This menu option opens the **'View Current User Details'** dialogue box, allows user to view their own details, except for their password.

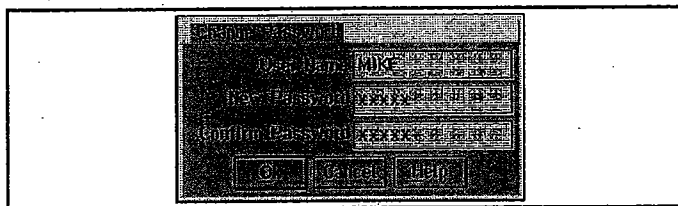


The dialogue box then has the following push buttons:

- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.

Change Password

This menu option opens the **'Change Password'** dialogue box, allows user to change their password.



The dialogue box has the following entry fields:

- **'User Name'** gives the present logged in user name.
- **'New Password'** requires the new password.
- **'Confirm Password'** requires the new password entered to be confirmed by re-typing the new password in this box. Failure to do this and the password will not be changed.

The dialogue box has the following push buttons:

- **'OK'** enters the new password for the user logged in.

Note: For this reason it is advisable to log out of the software when you are not present to avoid changes to your password.

- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.

Program

One of the unique features of the IsoPrime - EA software suite is the incorporation of a powerful programming facility. This enables the user to completely program the operation of sample preparation systems, control valves, stepper motors, read all analogue outputs from the instrument (including ion beams), and to set all digital registers available via the mnemonics (e.g. retuning the ion source from a program). In short the objective is to provide all the flexibility of an interpreted language like BASIC whilst retaining all the power and facilities of the operating system and the compiled source code. For details of programming see later sections of this manual.

The IsoPrime - EA software runs using these sequence files which allows the user to modify the way their system runs if desired.

Note: Care must be taken when altering sequences to avoid damage to the system.

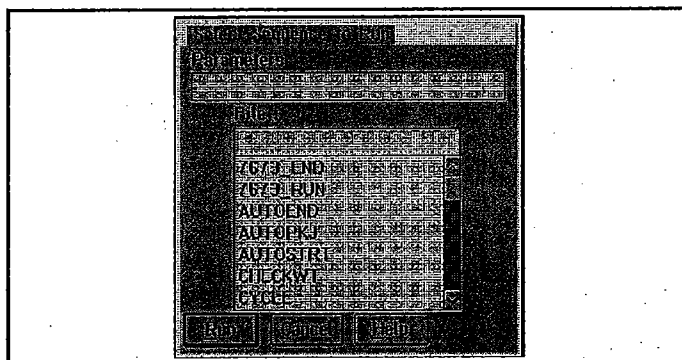
This menu gives the user the ability to use these programs individually from within the software.



The menu options are:

Run

This menu option will open the 'Select Sequence to Run' dialogue box, which executes a selected program.



This dialogue box contains a 'File' list box from which the program to be run is selected and a 'Parameters' entry field into which are put any parameters the program expects can be inputted. The dialogue box also has the following push buttons:

- 'Run' starts the selected program.
- 'Cancel' exits the dialogue box without taking any actions.
- 'Help' gives information on the operation.

IsoPrime - EA User Manual

Terminate

This menu option ends the execution of the currently running programs.

Hold

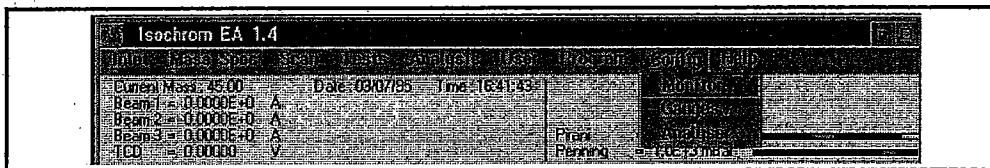
This menu option pauses the execution of the currently running programs.

Continue

This menu option resumes execution of the held program.

Config

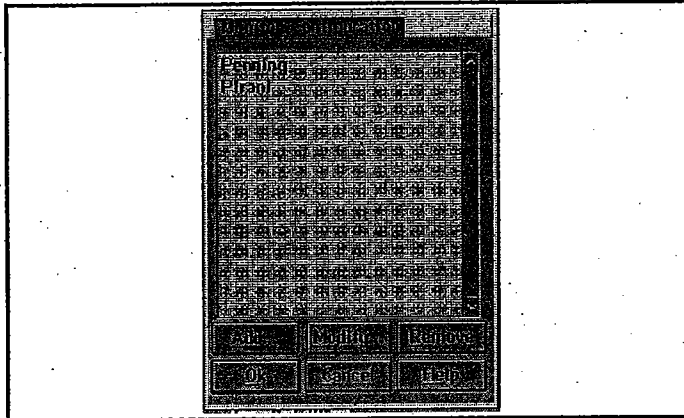
This menu gives the user the ability to decide what is monitored in the monitor window and to tell the software the number and type of vacuum gauges that are fitted to the system, thus allowing for further expansion.



The menu options are:

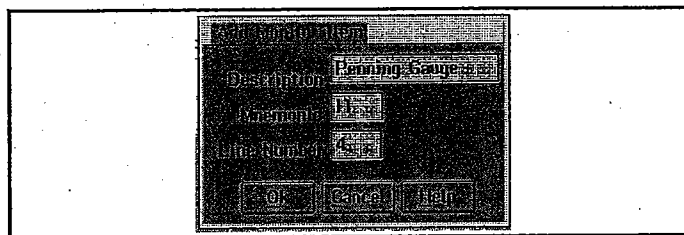
Monitor

This menu option will open the 'Monitor Configuration' dialogue box. This gives a list of which vacuum gauges and detectors are used on the system and to specify where on the screen they are displayed, either in the monitor window or in the various mimic diagrams on the main screen.



The dialogue box comprises of a list box of the various vacuum gauges and detectors specified and the following push buttons:

- 'Add' when selected opens the 'Add Monitor Item' dialogue box.



This allows for further gauges and detectors to be added to the system, if the system expands. The entry fields in this dialogue box expect the following information when adding a new gauges or detector:

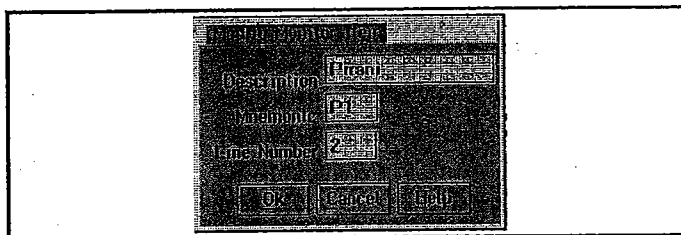
- 'Description' is the name of the device, and will appear in the 'Monitor Configuration' dialogue box.
- 'Mnemonic' is the mnemonic code that identifies the particular gauge or thermocouple in the software (see Table below for details).
- 'Line Number' is a number identifier that specifies where the gauge is displayed in the monitor window (up to 5 lines). If line '0' is chosen then the gauge or thermocouple is displayed only in the appropriate mimic diagram.

IsoPrime - EA User Manual

| Description | Mnemonic |
|-------------------------|----------|
| Penning Gauge | I1 |
| Analyser Backing Pirani | P1 |
| Turbo Speed | P4 |
| Magnet | MC |

This dialogue box has the following push buttons:

- **'OK'** adds the new gauge or detector to the list and updates the screen to include the new gauge, thermistor or thermocouple.
- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.
- **'Modify'** opens the **'Monitor Configuration'** dialogue box for the gauge or detector selected from the list box in the **'Monitor Configuration'** dialogue box.

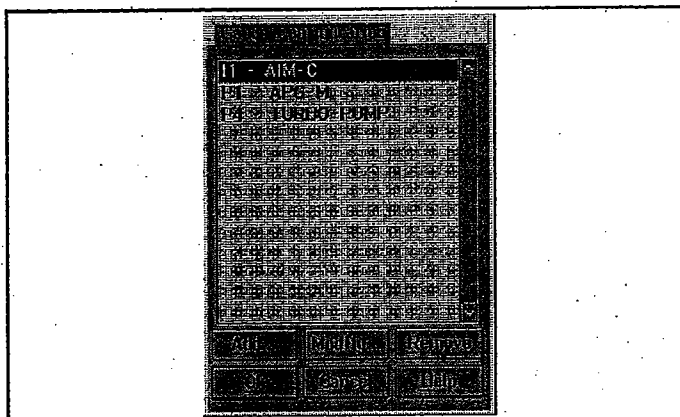


This dialogue box contains the details for the selected gauge, thermistor or thermocouple, and can be modified by changing any of the entry fields that have the same function as described above. The dialogue box has the following push buttons:

- **'OK'** will modify the gauge or detector details and update the screen.
- **'Cancel'** exits the dialogue box without taking any actions.
- **'Help'** gives information on the operation.
- **'Remove'** will remove the gauge or detector selected in the list box from the screen.
- **'OK'** will action any alterations and exit the dialogue box.
- **'Cancel'** will exit the dialogue box without taking any actions.
- **'Help'** gives information on the operation.

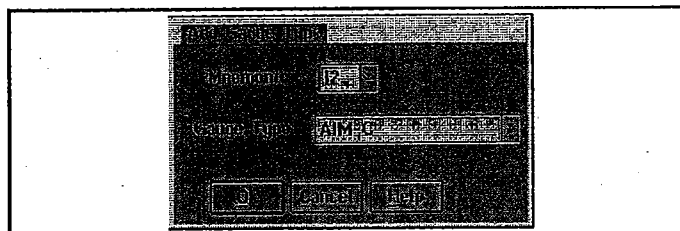
Gauge

This menu option will open the 'Gauge Configuration' dialogue box, This gives a list of which gauges are present on the system and their type, to enable the software to use the appropriate calibration routine, for pressure measurement.



The dialogue box comprises of a list box of the various vacuum gauges, specified and the following push buttons:

- 'Add' opens the 'Add Gauge type' dialogue box, which allows for further gauges to be added to the system in case of expansion.



The combo boxes in this dialogue box expect the following information when adding a new gauge:

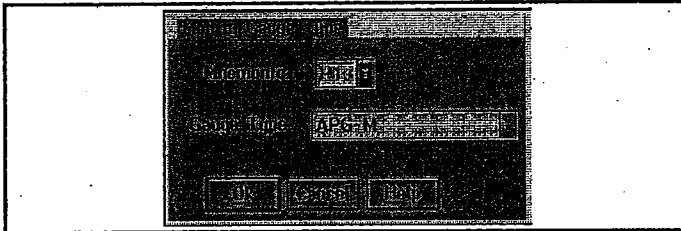
- 'Mnemonic' this is the mnemonic code that identifies the particular gauge in the software (see Table above for details).
- 'Gauge Type' this is where the gauge type is specified, the options for a particular mass spectrometer are listed in this combo box, details of which are listed in the Table below.

| Gauge Type | Description |
|------------|----------------------------|
| AIM-C | Penning Gauge (type AIM-C) |
| APG-M | Pirani Gauge (type APG-M) |
| Turbo Pump | Turbomolecular Pump Speed |

IsoPrime - EA User Manual

The dialogue box has the following push buttons:

- 'OK' will add the new gauge details to the list and update the software to include the new gauge.
- 'Cancel' exits the dialogue box without taking any actions.
- 'Help' gives information on the operation.
- 'Modify' opens 'Modify Gauge Type' dialogue box for the gauge selected from the list box in the 'Gauge Configuration' dialogue box.



This box will contain the details for the selected gauge, which can then be modified by changing the 'Gauge Type' combo box (for details of the combo box see above). It will be seen that the gauge mnemonic is grey as this cannot be modified. The dialogue box has the following push buttons:

- 'OK' will then modify the gauge details and update the software to include the modified gauge.
- 'Cancel' exits the dialogue box without taking any actions.
- 'Help' gives information on the operation.
- 'Remove' will remove the gauge selected in the list box from the screen.
- 'OK' will action any alterations and exit the dialogue box.
- 'Cancel' will exit the dialogue box without taking any actions.
- 'Help' gives information on the operation.

Help

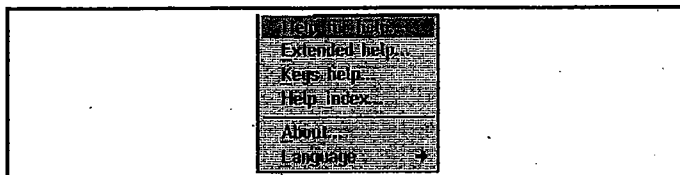
This menu allows the 'Help' facility of the IsoPrime - EA software to be accessed by the user. The 'Help' files provide a guide to the software as it is running and should be used in conjunction with this manual.

Note: As an alternative to using the 'Help' menu most windows have a 'Help' push button, which can be used to open the 'Help Window' for that section.

Help Window

Whenever a Help function is selected the information is display in the 'Help Window'. Within this window the IsoPrime - EA menu option functions are displayed as separate windows.

For further details on this window please consult the OS/2 Manual supplied with the system.



The menu options are:

Help for Help

This menu option when selected opens the '**Help for Help**' window. This details the way the help files can be accessed.

Keys help

This menu option when selected opens the '**Keys help**' window. This details the functions of the various keys e.g. Tab, Alt, etc.

Help index

This menu option opens the '**Help Index**' list box within the 'Help Window'.

From this list box the titles of the Help files are stored, therefore if for example, the file relating to Background Correction is to be found, step the list box (alphabetic order) to the section on Background Correction. Then by double clicking on the section required the Help file will then open the text available on the Background Correction so that it can be read.

About

This menu option when selected displays the '**application about box**' which details the application presently running.

Note: The 'Cancel' push button will exit this window.

Language

This menu option when selected opens a sub-menu, which enables the user to select the language for the help text.

Note: The language being used is indicated by the tick to the side of the menu items (this is not a menu on/off tick).

DP - Manual Data Processing

Introduction

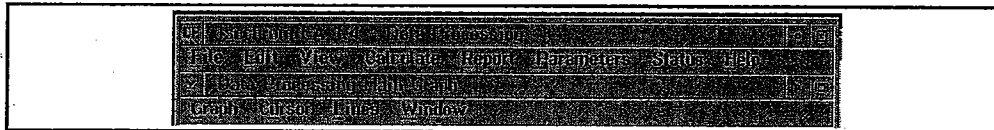
The DP software allows the manipulation of data acquired in the EA acquisition software. The software package provides the facility for the modification of the parameters setup for the automatic analysis which immediately follows data acquisition. In this chapter, the tools available for manual data processing are described. The EA software utilises the multitasking capability of the OS/2 operating system to allow manual data reprocessing of archived data during the acquisition of new data.

Data Storage

After data acquisition, the data collected from the collectors of the mass spectrometer and the TCD detector are stored in a Data Processing File (.DPF) file. Also included in the .DPF file, is information from the automatic data processing integration parameters (i.e. from the Events Parameters, Acquisition Parameters, and Analysis Parameters). After automatic analysis, a second file is generated. This is a parameter file, which is suffixed by .000 during storage. This contains such information as the times of HS and RG integration windows, sample peak limits, and background ratios etc., all of which can be modified in the DP software. Once modified, the .000 file is saved as a second parameter file suffixed by .001. This stores the revised parameters. The DP package accesses both the .000 and .001 files and provides opportunity to repeat the automatic data processing.

Accessing the DP software

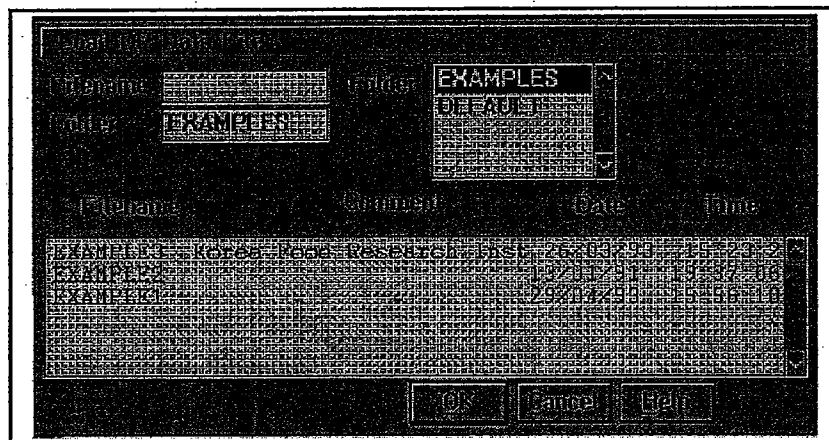
The DP software is accessed by double clicking on the DP icon in the EA folder. The DP main window will appear.



With the exception of File Menu and the Status and Help Facilities, the Menus remain inactive until either a .000 or .001 parameter file is loaded.

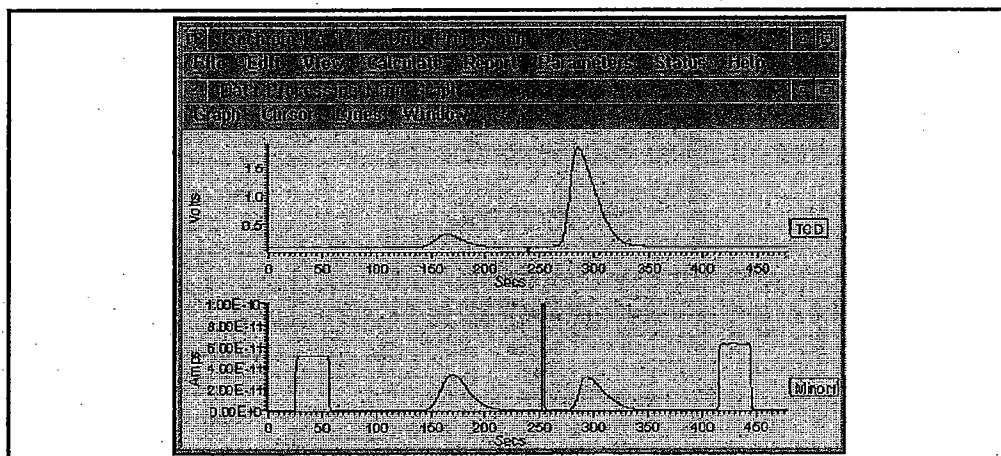
Loading Data

To load a DPF file into the DP software, click on the File menu and select the Load Data option. The Load DP Data File dialogue box will appear:



To load a data file:

1. Click in the desired folder name in the Folder List. The relevant folder name will appear in the Folder entry field, and the Filename List will be updated with the DPF files currently in that folder
2. Double click on the desired filename in the file list, or type the desired filename into the Filename entry field, and click on the OK button.
3. The system will automatically load that DPF file into the DP software. The DP main window will be updated with the DP graph window, in which will be drawn the TCD trace and the mass spectrometer collector traces thus:

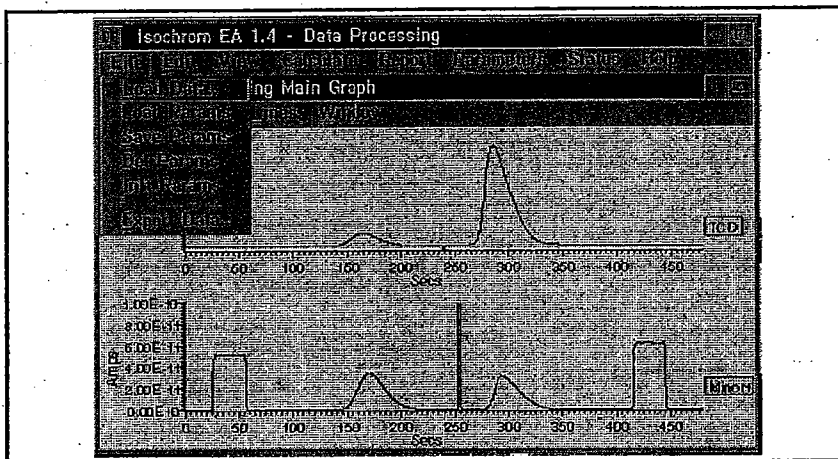


The DP main window menu bar

The menus displayed in the DP Main Window access facilities of the loading, manipulation and saving of analysis parameters, and for the printing of data.

File

The File Menu accesses facilities for the loading and storage of data and parameter files. The Menu is displayed below:



Load Data

This option accesses the Load DP Data File dialogue box described above, and allows loading of the *DPF* files into the *DP* software.

Load Params

Loads the .001 parameter file resulting from previous manual data processing activities. Once loaded, all the options on the main menu bar become active and the *DP* main graph is updated with the 2/1 and 3/1 ratio traces.

Save Params

Saves the current settings in a .001 file, overwriting any pre-existing .001 file in the process. Note when this option is chosen, the system asks for confirmation that the action be carried out.

Del Params

**** This facility is greyed out and is not accessible ****

Init Params

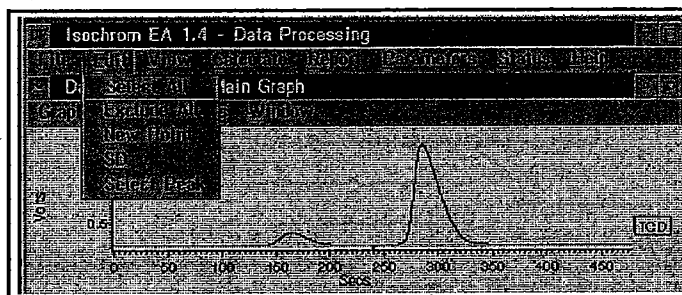
Loads the .000 parameter file generated after the automatic data processing.

Export Data

Access the Data Logger system for transport of data to spreadsheets.

Edit

The Edit Menu accesses facilities for the selection and deselection of sample, reference, background and zero points. It is necessary to request the 'Viewing' of these points before any manipulation from this menu can be done. This is achieved by using the 'View' menu described in the next section. The Edit menu is displayed below:



Select All

Reactivates all currently deactivated Sample Peaks, Background, Reference or Zero points displayed in the DP Graph Window

Deselect All

Deactivates all currently activated Sample Peaks, Background, Reference or Zero Points displayed in the DP Graph Window

New Point

Allows entry of new background points on the 2/1 and 3/1 ratio traces, and the collectors trace. On clicking on this command a hand icon with a pointing appears. In order to select a new point you must drag this pointer over the appropriate portion of the relevant trace.

SD

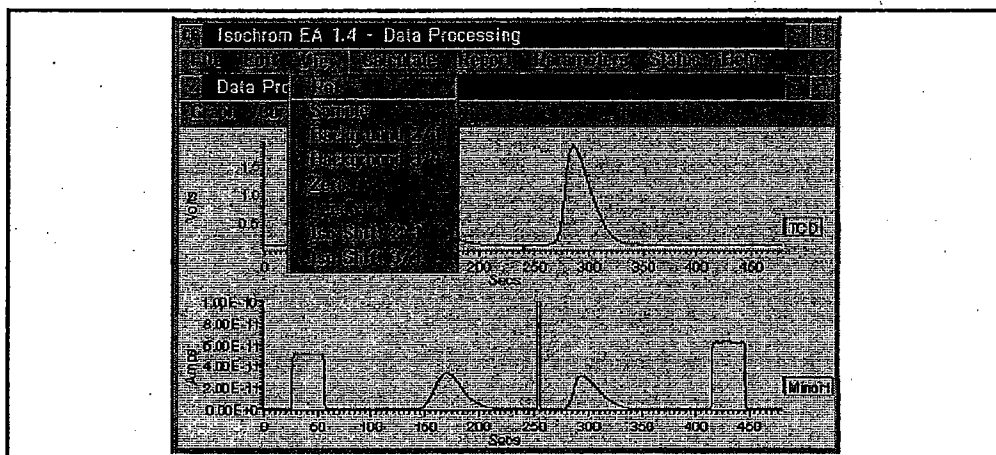
**** This facility is greyed out and is not accessible ****

Select Peak

Allows integration of unintegrated sample peaks that are smaller than the size thresholds specified in the Sample Integration Parameters.

View

The View Menu accesses options for the display of certain data points on the ratio and detector traces. The View Menu is displayed below:



Ref

Displays the 2/1 and 3/1 Reference ratio points and Reference polynomial curves on these ratio traces. It also displays the limits of integration for each reference gas pulse.

Sample

Displays the Sample Peak Limits and δ values on all integrated sample peaks on the collectors trace

Background 2/1

Displays the Background 2/1 points and polynomial curve on the 2/1 ratio trace, and the position of the background points (in grey) on the collectors trace. Active background points appear in green, whilst rejected background points are shown in red. Deactivated points turn to grey.

Background 3/1

Displays the Background 3/1 points and polynomial curve on the 3/1 ratio trace, and the position of the background points (in grey) on the collectors trace. Active background points appear in green, whilst rejected background points are shown in red. Deactivated points turn to grey.

Zero

Displays the Zero Points, and polynomial curve for each collector on the collectors trace. Active background points appear in green, whilst rejected background points are shown in red. Deactivated points turn to grey.

Iso Shift 2/1

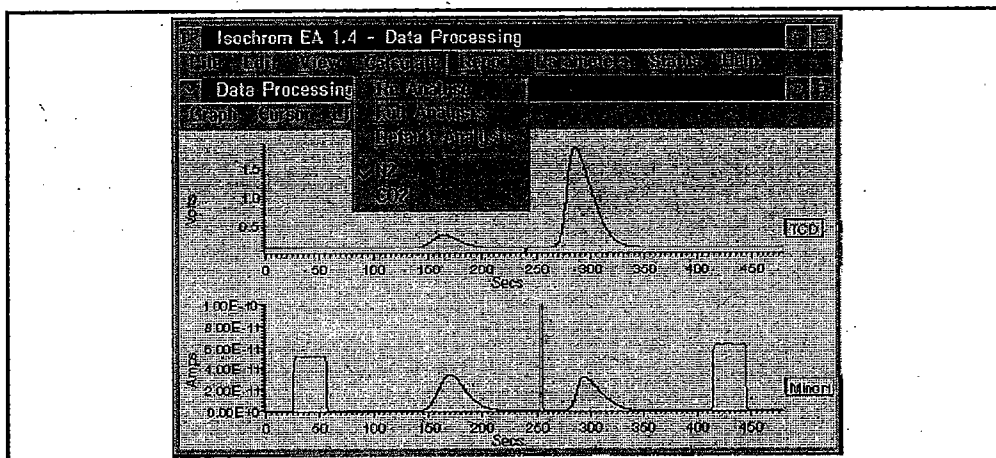
Displays the values for the isotopic shift, for each integrated peak, between the peak maxima on the Minor1 and Major collectors

Iso Shift 3/1

Displays the values for the isotopic shift, for each integrated peak, between the peak maxima on the Minor2 and Major collectors

Calculate

The Calculate Menu accesses facilities for the recalculation of δ values after modification of any analysis parameters. The Calculate Menu is displayed below:



Re Analyse

Performs a partial re-analysis of a data acquisition using the new values of any altered parameter(s).

Full Analysis

Performs a complete re-analysis of a data acquisition, including reference pulses, using the current parameters.

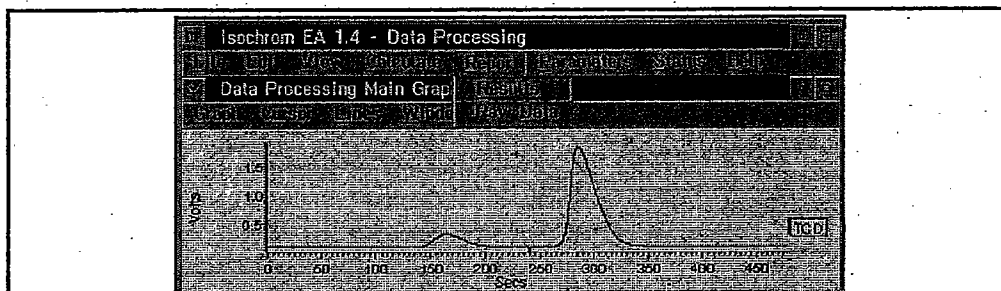
Default Analysis

Performs a complete analysis of data using the initial parameters stored for the AutoDP, prior to the analysis of the sample.

Note: Analysis is only performed on one gas species at a time as the backgrounds will be different. Selection of the gas species is done from the 'Calculate' menu

Report

The report menu accesses the data printing facility for *DP*. The Report Menu is displayed below:



Results

Prints the results relevant to the last 'Re-analyse' command in the default printer.

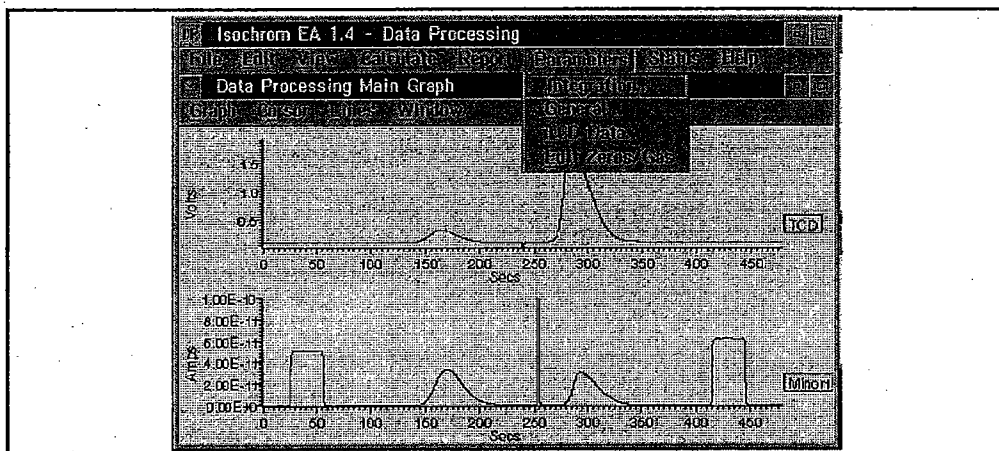
Raw Data

Accesses a facility for transporting the raw collector readings into spread sheets. On clicking on 'Raw Data', a confirmation is required to proceed. On confirmation, a pointer symbol appears. Click-and-drag to select the interval of interest. After selection the raw data is stored in a file named 'REPORT.TXT'. An example of the contents of the text file is shown below. This text file can be read into a spread sheet, or word-processing software, or printed to the printer.

| Data File Raw Data File - EXAMPLE2 Folder - | | | |
|---|-------|---------|---------|
| EXAMPLES | | | |
| Time | Major | Minor 1 | Minor 2 |
| 1357.1 | 504 | 541 | 445 |
| 1357.2 | 500 | 537 | 443 |
| 1357.3 | 497 | 531 | 438 |
| 1357.4 | 492 | 527 | 435 |
| 1357.5 | 490 | 525 | 433 |
| 1357.6 | 485 | 520 | 432 |
| 1357.7 | 483 | 517 | 428 |
| 1357.8 | 480 | 514 | 424 |
| 1357.9 | 477 | 509 | 421 |
| 1358.0 | 474 | 505 | 417 |
| 1358.1 | 472 | 504 | 418 |
| 1358.2 | 468 | 502 | 414 |
| 1358.3 | 467 | 499 | 411 |
| 1358.4 | 464 | 497 | 411 |
| 1358.5 | 462 | 493 | 408 |
| 1358.6 | 460 | 490 | 406 |
| 1358.7 | 458 | 489 | 404 |

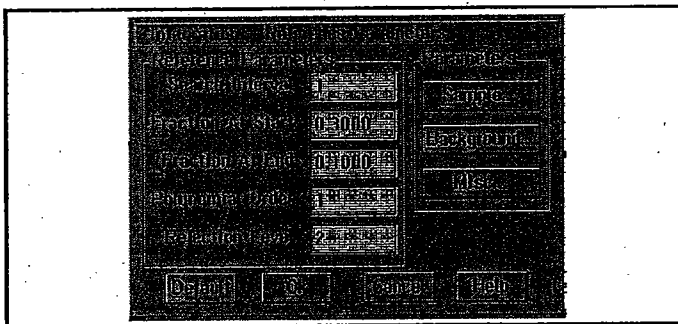
Parameters

The Parameters menu access all the integration parameters associated with the data processing algorithms. The Parameters menu is displayed below:



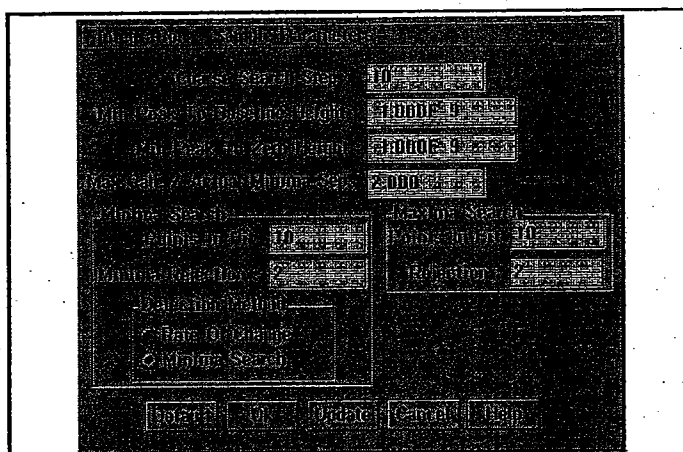
Integration Parameters

These parameters control the protocols for the data processing. The values initially displayed are stored in the .DPF file, and can be accessed after loading of the .000 parameter file by clicking on the 'Init Params' of the 'File' menu. Most of the parameters altered during data reprocessing are accessed from the integration parameters dialogue boxes. Click on the 'Integration' push button to access the **Integration - Reference Parameters'** dialogue box:

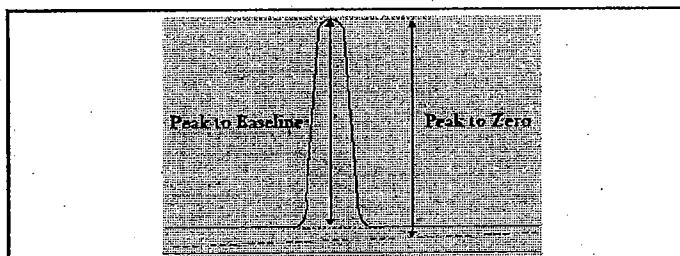


This dialogue box has the following panels:

- **'Reference Parameters'** panel has the following entry fields:
 - **'Search Interval'** is the number of data points used for searching for the reference pulses. **Note:** there are ten data points per second.
 - **'Fraction At Start'** is the fraction of the Reference pulse width at the start of the Reference Peak ignored during integration.
 - **'Fraction At End'** is the fraction of the Reference Pulse width at the end of the Reference Peak ignored during integration.
 - **'Polynomial Level'** is the order of the polynomial line drawn through the mean ratios of each reference pulse as a function of time. **Note:** it is highly recommended that this should be set to 1
 - **'Rejection Level'** is the number of standard deviations from the polynomial line outside of which individual reference points are rejected.
- **'Parameters'** panel controls the search, detection and size rejection parameters associated with the sample peak algorithms. The panel has the following push buttons:
 - **'Sample'** accesses the 'Integration - Sample Parameters' dialogue box. This dialogue box has the following features:



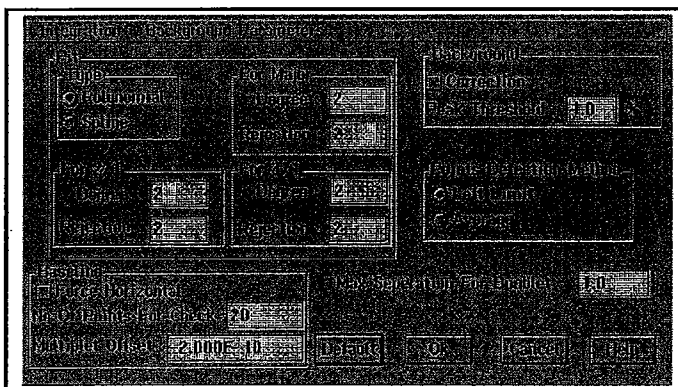
- 'Coarse Search Step' entry field is the number of points per step in the initial coarse search for sample peaks which should be increased to approximately 13 if peaks are missed.
- 'Min Peak To Baseline Height' entry field peaks of baseline to peak maxima height less than this value are not integrated
- 'Min Peak To Zero Height' entry field peaks of zero line to peak maxima less than this value are not integrated (see figure below)



- 'Max Calc / Actual Minima Sep.' compares the positions of the minima from the coarse search, MINIMA SEPARATION and the polynomial fit. If these are within the specified number of scans the peak limit is placed at the measured minima, otherwise the polynomial minima is used.
- 'Minima Search' panel has the following features:
 - 'Points In Fit' entry field is the number of points used in drawing a second order polynomial, around each coarse minimum, to find the exact peak limits.
 - 'Minima Rejection' entry field is the rejection level, in standard deviations, of the polynomial fit used in detecting the peak minima.

IsoPrime - EA User Manual

- 'Maxima Search' panel has the following features:
 - 'Points In Fit' entry field is the number of points used in the vicinity of the peak maximum to calculate the exact position of that maximum.
 - 'Rejection' entry field is the rejection level, in standard deviations, of the 2nd order polynomial fit used in detecting the peak maximum.
 - 'Detection Method' panel. **** This facility is greyed out and is not accessible. ****
 - 'Rate of Change' radio button **** This facility is greyed out and is not accessible. ****
 - 'Minima Search' radio button detects the preliminary peak limits by performing a coarse search for the minima on the left and right of a peak maximum.
- The 'Integration - Sample Parameters' dialogue box has the following push buttons:
- 'Default' updates the reference integration parameters to the default values. Note: default values are displayed above and are highly recommended.
 - 'Ok' saves any changes made in any of the integration parameters and closes the window
 - 'Cancel' closes the window without saving any changes
 - 'Help' accesses the help files for this dialogue box.
- 'Background' push button accesses facilities for fitting the background composition line during background subtraction. This facility is not normally used with the Elemental Analyser preparation system. It is however described in full here, in case this facility should be needed in very exceptional circumstances. Some facilities are accessed for modelling the shape of the base of each sample peak. Selecting this push button opens the 'Integration - Background Parameters' dialogue box. This dialogue box has the following panels:



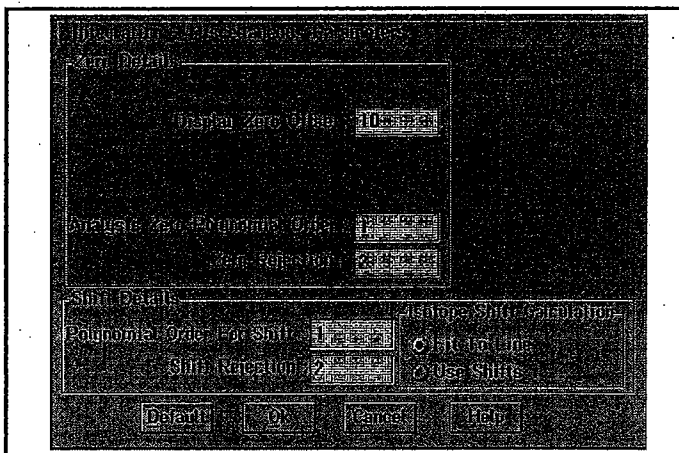
- 'Background' panel has the following features:
 - 'Correction' tick box activates background subtraction procedures during Auto DP
 - 'Peak Threshold' entry field sets the trigger level for background subtraction e.g. if background height is more than 1% of total peak height then a background subtraction is performed on this particular

height then a background subtraction is performed on this particular peak. This facility enables the user to perform background subtraction for the sample peaks eluting on a significant background, whilst allowing only a zero subtraction on peaks on small backgrounds within the same chromatograph.

- **'Points Detection Method'** panel has the following features:
 - **'Left Limit'** radio button measures the background ratios on the 2/1 or 3/1 traces at the left limit time of each peak
 - **'Average'** radio button measure the background composition on the 2/1 and 3/1 traces as an average of the ratios at the left and right time limits of each sample peak.
- **'Fit'** panel has the following features:
 - **'Type - Polynomial'** radio button selects, as a polynomial, the type of curve drawn through the background composition points.
 - **'Type - Spline'** **** This facility is greyed out and is not accessible. ****
 - **'For Major - Degree'** **** This facility is greyed out and is not accessible. ****
 - **'For Major - Rejection'** **** This facility is greyed out and is not accessible. ****
 - **'For 2/1 - Degree'** sets the order of the polynomial curve drawn through the 2/1 background points (up to order 6 is allowed).
 - **'For 2/1 - Rejection'** is the rejection limit (in standard deviations) for the above polynomial.
 - **'For 3/1 - Degree'** sets the order of the polynomial curve drawn through the 3/1 background points (up to order 6 is allowed).
 - **'For 3/1 - Rejection'** is the rejection limit (in standard deviations) for the above polynomial.
- **'Baseline'** panel has the following features:
 - **'Force Horizontal'** tick box draws the doublet/multiplet baseline as a horizontal line from the lower multiplet limit, rather than joining the left and right multiplet limits.
 - **'No of Points For Check'** entry field is the number of points used to check that the peak baselines do not cut the actual beam intensity traces. If beam traces are cut, a horizontal fit is forced.
 - **'Multiplet Offset'** **** This facility is greyed out and is not accessible. ****
 - **'Max Separation For Doublet'** entry field marks as 'in a multiplet' any peak limits which are within this number of scans of the peak limits of an adjacent peak
- The **'Integration - Background Parameters'** dialogue box has the following push buttons:
 - **'Default'** updates the reference integration parameters to the default values. Note: default values are displayed above and are highly recommended.

IsoPrime - EA User Manual

- 'OK' saves any changes made in any of the integration parameters and closes the window
- 'Cancel' closes the window without saving any changes
- 'Help' accesses the help files for this dialogue box.
- 'Misc' push button accesses parameters which allow programming of the 'Zero' function and monitoring of the Isotopic Shift values. Selecting 'Misc', opens the 'Integration - Miscellaneous Parameters' dialogue box. This dialogue box has the following panels:



- 'Zero Details' panel has the following features:
 - 'Number Of Points For Zero' entry field. It is the number of data points which are to be used in order to measure the beam zeros. Data points are collected every 100 ms.
 - 'Display Zero Offset' entry field is the number of scans after the start of data acquisition after which a zero is taken. This reading used only to give approximate displayed 2/1 and 3/1 ratio traces during acquisition, and takes no part in the Auto DP
 - 'Analysis Zero Start Offset' entry field Defines the number of data points counting from the start of the acquisition after which the beam zeros will be measured.
 - 'Analysis End Offset' entry field Defines the number of data points counting from the end of the acquisition up to which the zeros will be measured.
 - 'Analysis Zero Polynomial Order' entry field describes the order of the polynomial drawn through the 'Analysis Zero Points' described in ZERO DETAILS above. It is strongly recommended that this should be set to 1.
 - 'Zero Rejection' entry field is the rejection level (in standard deviations) in the above polynomial fit.
- 'Shift Details' panel has the following features:
 - 'Polynomial Order For Shift' entry field describes the polynomial order used in the generation of the Shift Curve described above.

IsoPrime - EA User Manual

- 'Shift Rejection' entry field is the rejection level (in standard deviations) for points in the above line fit.
- 'Isotope Shift Calculation - Use Shifts' radio button disables the 'Fit to Line' feature. Individually measured peak - values each peak are used in the isotopic shift calculation
- 'Isotope Shift Calculation - Fit To Line' plots a line through the isotopic shift values for each peak as a function of time. The isotope shift value used for each peak is then read from the line rather than the individually measured peak value.

The 'Integration - Miscellaneous Parameters' dialogue box has the following push buttons:

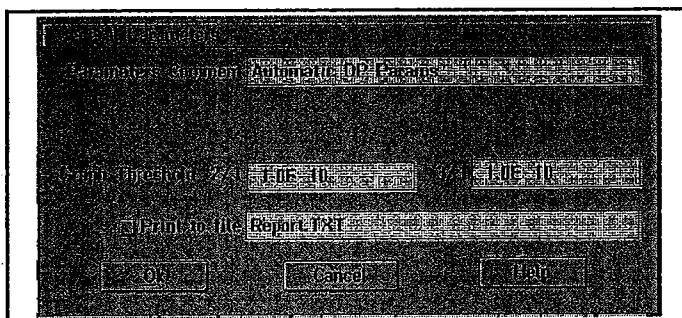
- 'Default' updates the reference integration parameters to the default values. Note: default values are displayed above and are highly recommended.
- 'OK' saves any changes made in any of the integration parameters and closes the window
- 'Cancel' closes the window without saving any changes
- 'Help' accesses the help files for this dialogue box.

The 'Reference Parameters' dialogue box has the following push buttons:

- 'Default' updates the reference integration parameters to the default values. Note: default values are displayed above and are highly recommended as a first attempt on an unknown mixture.
- 'Save' saves any changes made in any of the integration parameters and closes the window
- 'Cancel' closes the window without saving any changes
- 'Help' accesses the help files for this dialogue box.

General

The 'General Parameters' dialogue box accesses those parameters that are external to the data acquisition. The General Parameters dialogue box is displayed below. The dialogue box has the following features:



- 'Parameters Comment' entry field will accept up to 32 characters, to enable any comment to be recorded to store along side the analysis.

IsoPrime - EA User Manual

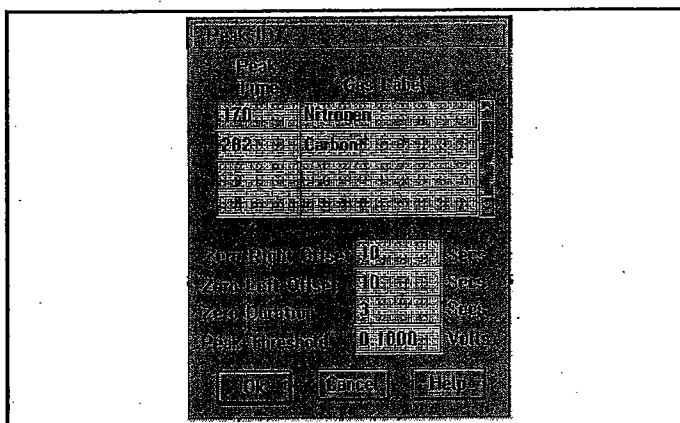
- **'Graph Threshold 2/1 and 3/1'** The entries in these two fields give the values of the major ion currents in Amps, below which the true 2/1 and 3/1 ratio traces will not be displayed. Instead a straight line will appear with an offset equal to the ratios for the reference gas pulses as acquired in the previous run..
- **'Print To File'** tick box when selected, writes the results to the text (.txt) file specified in the entry field.

The **'General Parameters'** dialogue box has the following push buttons:

- **'OK'** saves any changes made in any of the integration parameters and closes the window
- **'Cancel'** closes the window without saving any changes
- **'Help'** accesses the help files for this dialogue box.

TCD DATA

The TCD data window provides the facility to specify the parameters that are necessary for the integration of peaks acquired on the TCD, from which a calculation of the elemental composition of a sample can be made.



The peaks for which integration has to performed are specified in the upper part of this window, where the approximate time for the top of these peaks are entered. Associated with each peak a gas label may be declared. The lower half of this window provides the information needed for the integration of the TCD peaks. in the fields provided, it is necessary to declare how the zeros are to be taken and the peak thresholds below which no integration will be performed. This is to ensure that within the integration window, noisy portions are not identified as peaks.

- The **'Zero Right Offset'** indicates the time at which the right zero has to measured relative to the end of the run.
- The **'zero left offset'** indicates the time at which the left zero has to be measured relative to the start of the run.
- The **'zero duration'** indicates the length of time for which the zeros have to be measured. This time period applies to both the right and left zeros. It is not possible to declare a duration time for one of the zeros and a different duration time for the other zero.

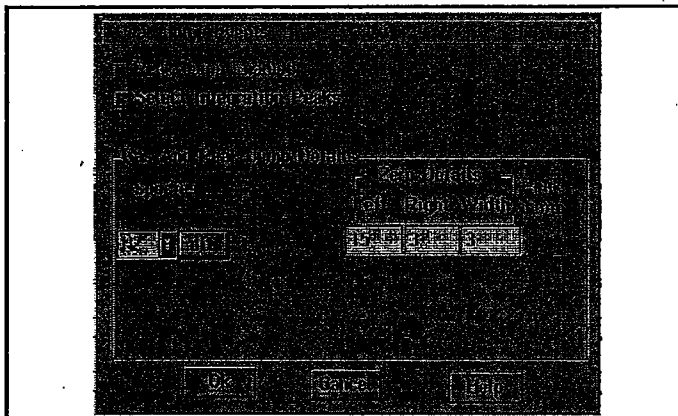
IsoPrime - EA User Manual

Notes:

All the times declared are in seconds.

If only the left zero is to be declared, then the right zero entry field must be left blank. Similarly if only the right zero is to be declared, then the left zero entry has to be left blank. If no zero subtraction is required, then both sets of entries must be left blank.

Edit Zeros/Gas

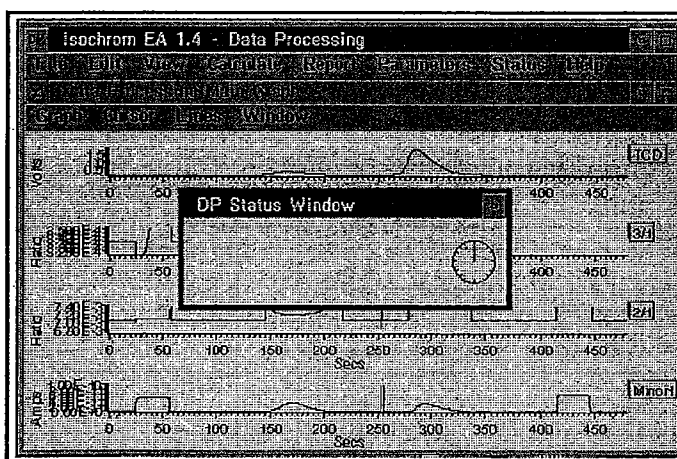


This window allows the editing of the information relating to zeros, to be changed, if it is felt necessary, in order to obtain a calculation that is more valid.

- The zero details are all measured in seconds.
- The left zero is measured from the start of the acquisition period.
- The right zero is measured from the end of the acquisition period.
- The width refers to the duration period (in seconds) during which zeros are taken. This duration period is common to both the left and the right zeros. It is not possible to declare one time period for one of the zeros and another time period for the other zero.
- The Edit push button gives access to the calibration details for the reference gas. (This window was described in a previous section)

Status

The status menu accesses the Status Window, which displays the current status of any calculations or processing activities. The Status Window is shown below:



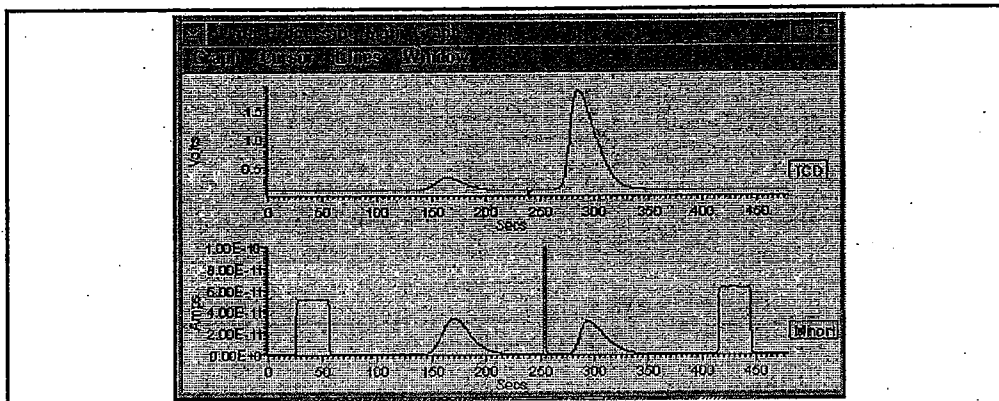
The Status Window can be minimised to an icon. If the Status Window is not visible, click on the Status Menu. The Window should appear. If the icon is displayed, double - click on the icon to restore the Window.

Help

**** This facility is greyed out and is not accessible. ****

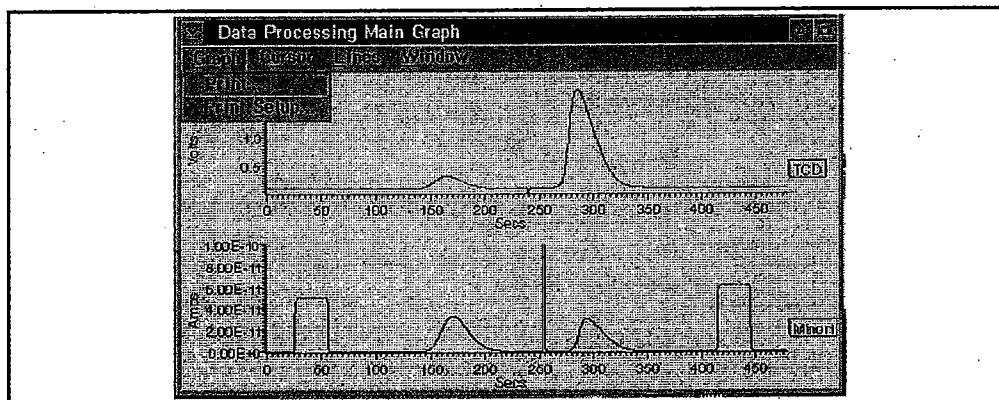
DP Graph Window Menu Bar

The ion beams, TCD and 2/1 and 3/1 ratio traces are displayed in the Main Graph Window. The menu bar at the top of the graph window provides facilities for manipulation of the graphic displays in the graph window. The Graph Window menu bar is shown below:



Graph

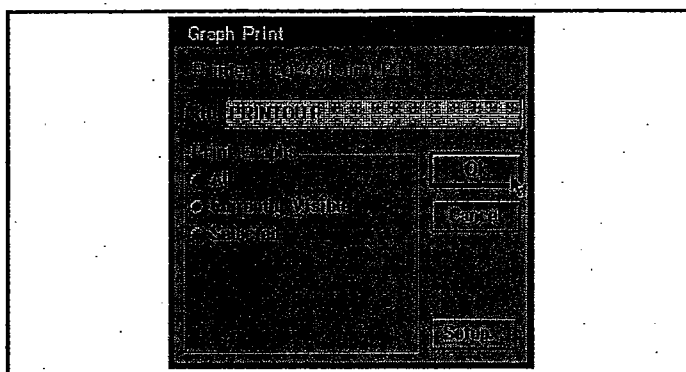
The Graph Menu provides a facility for printing of the traces displayed in the Graph Window. Before using the print options, it is necessary to set up the appropriate printer driver on the OS/2 desktop. The Graph Menu is displayed below:



This menu option has the following commands:

- 'Print'

This menu option opens the 'Graph Print' dialogue box.



This dialogue box allows the print out to be given a title in the 'Title' entry field and to select the graphs to be printed using the radio buttons in the 'Print Graphs' section. The radio buttons are:

- 'All' when selected prints all the graphs selected from scan.
- 'Currently Visible' when selected will print all the graphs in the scan window.
- 'Selected' when selected opens a list box from which graphs to be printed can be selected, by clicking on the required graph title.

The dialogue box also has the following push buttons:

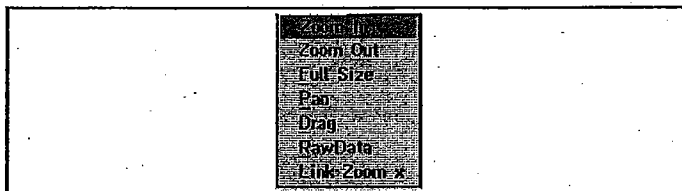
- 'OK' will then start the print out.
 - 'Cancel' push button exits the dialogue box without taking any actions.
 - 'Setup' will open the 'Select Printer' dialogue box as described below.
- 'Print Setup'

This menu option opens the 'Select Printer' dialogue box. This allows the type of printer to be printed to, to be chosen. A list of available printers appears in the list box

The dialogue box has the following push buttons:

 - 'OK' will specify the printer selected in the list box as the preferred printer until changed (by selecting a different printer).
 - 'Cancel' push button exits the dialogue box without taking any actions.
 - 'Setup' will open the selected printers 'Job Properties' dialogue box, from which the print out details can be edited. For details of the 'Job Properties' dialogue box see OS/2 Manual.
 - 'Help' push button gives information on the operation.

Cursor



This menu controls the behaviour of the mouse pointer within the graphical display area. The commands all act with a 'Menu Tick' function and are:

- **'Zoom In'**

When chosen the mouse cursor becomes a magnifying glass symbol. By dragging the magnifying glass over an area of the scan it can be examined in greater detail. If required the user can 'Zoom In' in several stages.

- **'Zoom Out'**

By clicking on a 'Zoom In' graph after selecting 'Zoom Out', the plot will go back to the size previously displayed prior to a 'Zoom In', this can be done several times until the graph is back to full scale use.

- **'Full Size'**

This will return a graph to full scale, simply by clicking on the plot.

- **'Pan'**

**** This facility is greyed out and is not accessible. ****

- **'Drag'**

When Drag is selected, the mouse can be used to pick up and move any vertical cursor line used to showing on a graph. For example, it is used for moving the limits of integration and selecting the users own limits. In the DP main graph window this option also enables points to be switched OFF and reactivate them if necessary. It is used for deselecting background points on both the 2/1 and 3/1 ratio traces and also to deselect sample peaks thus eliminating these peaks from integration.

- **'Raw Data'**

Raw Data mode provides an additional vertical cursor, which can be used in conjunction with the drag facility to examine the raw data in a small window at the bottom left of the screen.

1. Select Link Zoom
2. Click on graph A
3. Click on Graph B

This menu contains a list of the lines available for display. The text in the menu is the label entered in the Scan Setup dialogue box. By ticking lines on or off from this menu the display window can be simplified.

The commands all act with a 'Menu Tick' function and are:

- **'Cascade'**

- **'Tile'**

DP - Manual Data Processing

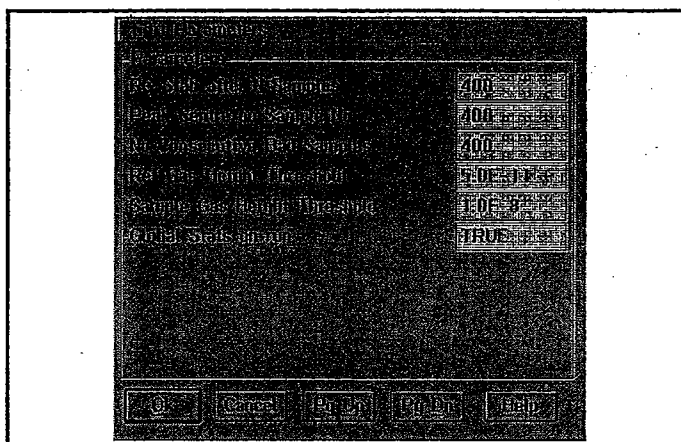
Parameter Files

The following pages of this section will give a brief description of the parameter files, for reference. For details of how to edit them see earlier in this section and for more in depth details of their function see later sections on the individual preparation systems.

The parameters for the system are set during the test and installation period and are tailored to the system, however, if desired, they can be edited to satisfy special requirements.

Autorun Procedure Settings Parameter File

The Autorun Procedures Settings parameters are shown below.



RG Stab after N Samples

Specifies the number of sample analyses (N) after which the Rctest method is activated to perform a reference gas stability run.

Peak Centre on Sample No

Specifies the number of samples (N) after which a peak centre is performed by running the Centre scan file.

No of Consecutive Bad Samples

Specifies the number of consecutive rejected samples after which an autorun will be aborted. The sample is rejected if the sample peak has a peak height less than the value entered for the Sample Gas Height Threshold parameter

Reference Gas Height Threshold

Specifies the reference gas major beam intensity below which a centre scan is triggered. The reference gas height check is performed at the end of every sample.

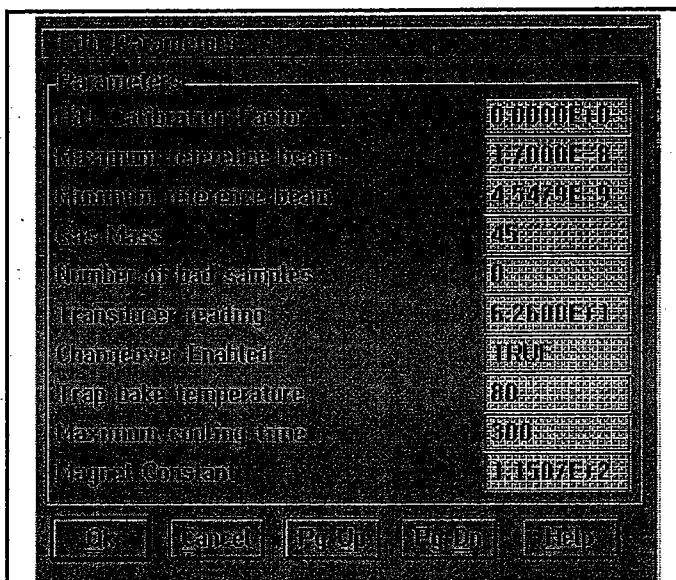
Sample Gas Height Threshold

Specifies the sample peak maximum major beam intensity below which a sample peak is rejected.. The number of consecutive rejected samples after which the autorun is aborted is specified in the No of Consecutive Bad Samples.

Global Stats on Run

**** This facility is greyed out and is not accessible. ****

Constants Parameter File



H3+ Calibration Factor

Not used on IsoPrime.

Maximum reference beam

Not used on IsoPrime.

Minimum reference beam

Not used on IsoPrime.

Gas Mass

The current axial mass is stored here (also displayed in the monitor window).

Number of bad samples

Not used on IsoPrime.

Transducer reading

Not used on IsoPrime.

Changeover Enabled

Not used on IsoPrime.

Trap bake temperature

Not used on IsoPrime.

Maximum cooling time

Not used on IsoPrime.

Magnet Constant

General Mass Spectrometer equation used when HT peak jumping and is updated when the peak is identified.

HT Settle Time

This is the time allowed after a HT peak jump for the system to settle prior to data being measured.

Hall Probe Enabled

This location stores whether the Hall Probe has been enabled.

Use Mass Cal With Hall Probe

This location stores whether the mass calibration used the Hall Probe.

Reference bellows maximum

Not used on IsoPrime.

Sample bellows maximum

Not used on IsoPrime.

Amplifier Zero Channel 0

The location of the main analyser Low 1 collector amplifier zero is stored.

Amplifier Zero Channel 1

The location of the main analyser Axial collector amplifier zero is stored.

Amplifier Zero Channel 2

The location of the main analyser High collector amplifier zero is stored.

Amplifier Zero Channel 3

The location of the main analyser Low 2 collector amplifier zero is stored.

Amplifier Zero Channel 4

Not used on IsoPrime

Amplifier Zero Channel 5

Not used on IsoPrime

Amplifier Zero Channel 6

Not used on IsoPrime

Amplifier Zero Channel 7

Not used on IsoPrime

Amplifier Zero Channel 8

Not used on IsoPrime.

Amplifier Zero Channel 9

Not used on IsoPrime.

Amplifier Zero Channel 10

Not used on IsoPrime

Amplifier Zero Channel 11

Not used at on IsoPrime

Resistor Gains Channel 0

The location of the main analyser Low 1 collector amplifier resistor value is stored. (5E+8)

Resistor Gains Channel 1

The location of the main analyser Axial collector amplifier resistor value is stored. (5E+10)

Resistor Gains Channel 2

The location of the main analyser High collector amplifier resistor value is stored. (1E+11)

Resistor Gains Channel 3

The location of the main analyser Low 2 collector amplifier resistor value is stored. (5E+8)

Resistor Gains Channel 4

Not used on IsoPrime.

Resistor Gains Channel 5

Not used on IsoPrime.

Resistor Gains Channel 6

Not used on IsoPrime

Resistor Gains Channel 7

Not used on IsoPrime.

Resistor Gains Channel 8

Not used on IsoPrime.

Resistor Gains Channel 9

Not used on IsoPrime.

Resistor Gains Channel 10

Not used on IsoPrime

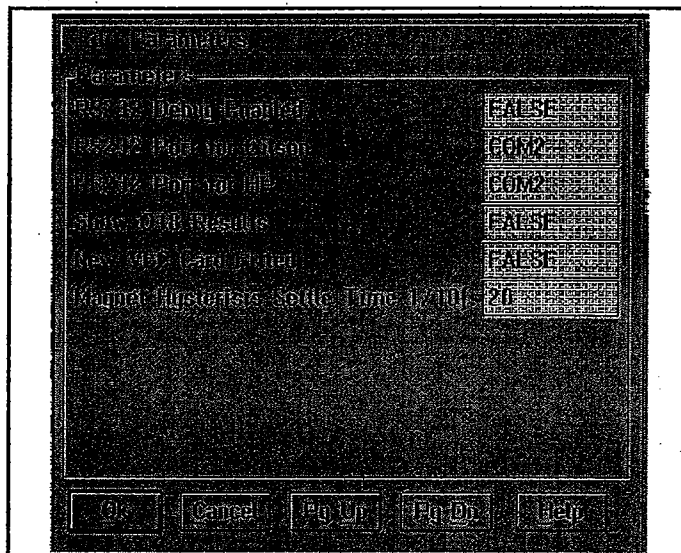
Resistor Gains Channel 11

Not used on IsoPrime

Current Rack Parameters

This window includes the various parameters that are required to define the geometry and characteristics of autosamplers and autoinjectors, which may be used in conjunction with the sample preparation systems capable of being interfaced to the mass spectrometer. With the Elemental Analyser system, this window is not used.

General Parameters



RS232 Debug Enabled

When 'TRUE' is entered, any error messages associated with operation of the autoinjector are printed in the Message window

RS232 Port for Autoinjector

Specifies the computer COM port being used to communicate with the autoinjector

Show O18 Results

When 'TRUE' is entered the $\delta^{18}\text{O}$ values for each peak are printed on the results printout.

New VFC Card Fitted

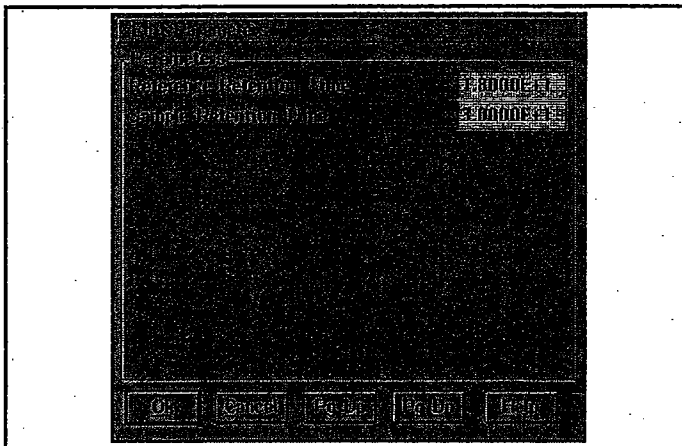
Specifies the generation of VFC card currently fitted in the system controller. This is manifest as a x10 scaling factor on the FID trace.

HP Rack Parameters

This not used with the EA prep system.

Retention Times Parameter File

The retention time records the sample and reference gas retention time parameters that are global constants in the Method Setup facility.



Reference Retention Time

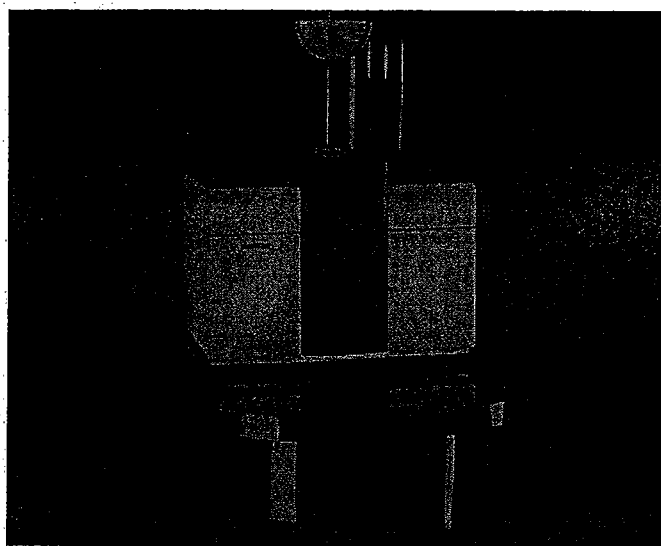
Specifies the delay between the RG or RN valve action, and a complete response from the mass spectrometer.

Sample Retention Time

Specifies the increased retention time resulting from a sample peak passing through the preparation interface.

IsoPrime EA User Manual
Code No 6666588
Issue 1a

Section 6



Operating Instructions



IsoPrime-EA User Manual

| | |
|---|-----------|
| Introduction | 7 |
| Mains voltage configuration | 7 |
| Rotary pump..... | 7 |
| IsoPrime cubicle..... | 7 |
| IsoPrime system controller | 7 |
| Turbomolecular pump controller | 7 |
| Electromagnet power supply | 8 |
| Bakeout power supply | 8 |
| Switching the Electrical Circuits..... | 9 |
| Switching-on the Rotary pump | 9 |
| Switching-on the IsoPrime..... | 9 |
| Switching on the EA | 9 |
| Switching off the Electrical Supplies | 10 |
| Starting The IsoPrime-EA Software..... | 10 |
| Starting Up The System Software | 10 |
| Starting Up The Data Processing (DP) Software | 11 |
| Logging In..... | 12 |
| Logging Out..... | 12 |
| Configuring the system..... | 12 |
| Closing Down The Software..... | 12 |
| Pumping The Mass Spectrometer | 13 |
| To start the Analyser Rotary Pump..... | 13 |
| Starting the turbomolecular pump | 14 |
| The vacuum gauges..... | 14 |
| Prep System Operations..... | 14 |
| NA2500 Set-up | 14 |
| Packing the Furnace Tubes..... | 15 |
| Setting the gas flows | 15 |
| Time and Temperature Set-up | 16 |
| Setting the Sample Drop Time..... | 16 |
| TCD output signal..... | 17 |
| NA2500 start / stop..... | 17 |
| Setting Up the Reference Gas Injector | 17 |
| Single injector..... | 18 |
| Dual injector | 18 |

| | |
|---|-----------|
| Setting the Mass Spectrometer..... | 19 |
| Introduction..... | 19 |
| Admitting gas into the mass spectrometer | 19 |
| Switching the Ion Source ON | 20 |
| Coarse tuning the Ion Source..... | 20 |
| Recommended Values..... | 20 |
| Recommended Source Tuning Procedure | 21 |
| Checking the coincidence | 21 |
| Cleanliness of the vacuum and Leak checking | 22 |
| Cleanliness of the vacuum..... | 22 |
| Leak checking | 23 |
| Checking the system prior to running a sample..... | 24 |
| Backgrounds..... | 24 |
| Acceptable background levels..... | 24 |
| Fine tuning the Ion source..... | 25 |
| Stability of the instrument..... | 27 |
| Evaluation of stability using 'Time scans' | 27 |
| The Reference Gas Stability Test..... | 28 |
| Linearity | 31 |
| Sample running | 32 |
| Sample chromatography..... | 33 |
| Run method file timings..... | 33 |
| N2 Run method file..... | 33 |
| CO2 Run method file | 37 |
| NC Run method file | 37 |
| BlankN method file..... | 39 |
| BlankC method file | 40 |
| BlankNC method file | 40 |
| Starting a single run | 40 |
| Selecting the single run option | 40 |
| The final report | 41 |
| Introduction to manual data processing | 42 |
| Loading the single run data file | 43 |
| Checking the method file parameters..... | 43 |
| Closing the DP Software..... | 44 |

IsoPrime-EA User Manual

| | |
|--|-----------|
| Setting up an autorun..... | 44 |
| Creating a new autorun file..... | 44 |
| Autorun options | 45 |
| Autorun batch summary report | 48 |
| Spreadsheet transfer of summary report | 50 |
| Batch DP post data analysis | 50 |
| Batch DP options | 50 |

IsoPrime-EA User Manual

Introduction

This section of the manual describes the step by step actions needed to get the IsoPrime and EA interface up and running from complete 'shut down'. If the instrument is in a state different to complete 'shut down', please start with the section which best represents the status of the system. This section therefore will guide the user from electrical switch on of the instrument to running a real sample.

From complete 'shut down' the entire sequence of actions is :

1. Power up the entire system.
2. Start up the software.
3. Pump the mass spectrometer
4. Set up the reference gas injector
5. Set up the mass spectrometer
6. Set up the EA and its interface
7. Perform preliminary tests to check the performance of the entire system
8. Run a standard mixture i.e. UREA
9. Run a real sample
10. Reanalyse data, if necessary

Mains voltage configuration

Rotary pump

The rotary pump may be configured for operation at 110Vac or 220Vac.

The configuration is accomplished by the operation of a rocker switch located under the top cover of the rotary pump.

The current voltage setting is displayed on a reversible plate visible through the side of the rotary pump.

The instructions given in the manufacturer's manual should be followed to change the voltage setting.

IsoPrime cubicle

IsoPrime system controller

The IsoPrime system controller contains a switched mode power supply that will operate over a range of input voltages. The mains supply should be within the range 100-300Vac for the power supply to operate.

No configuration of the power supply is required for operation at different voltages. The power supply will automatically adjust to a mains voltage within its operating range.

Turbomolecular pump controller

The turbomolecular pump controller contains a switched mode power supply that will operate over a range of input voltages. The mains supply should be within the range 100-300Vac for the power supply to operate.

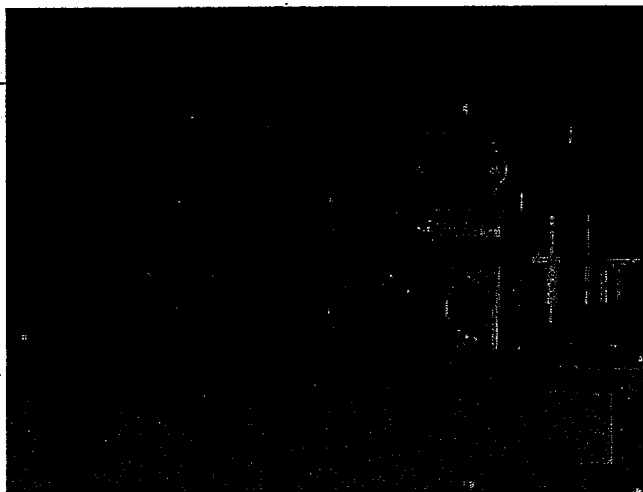
No configuration of the power supply is required for operation at different voltages. The power supply will automatically adjust to a mains voltage within its operating range.

Electromagnet power supply

The electromagnet power supply will operate on 110Vac or 230Vac mains input.

The voltage may be selected using the switch mounted on the rear of the electromagnet power supply next to the mains input.

Voltage selector
switch



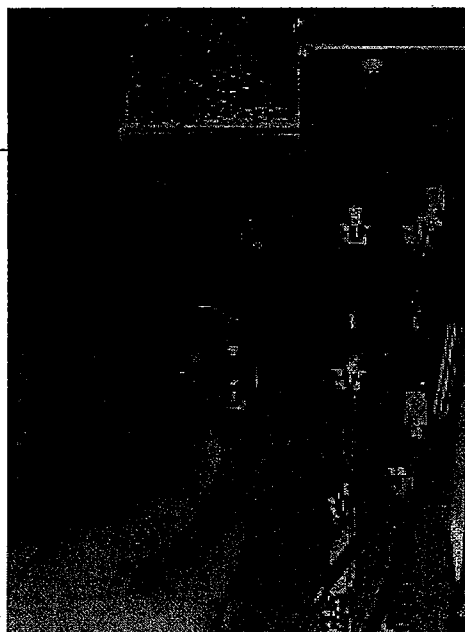
Electromagnet power supply voltage selector switch

Bakeout power supply

The bakeout power supply will operate on 110Vac or 230Vac mains input.

The voltage may be selected using the switch mounted on the front of the bakeout power supply next to the mains input.

Voltage selector
switch



Bakeout power supply voltage selector switch

Switching the Electrical Circuits

This section of the manual describes switching on/off the electrical circuit of the IsoPrime. The electrical circuit is separated into two halves:

1. IsoPrime.
2. Rotary pump.

Each circuit has its own mains cable, which are connected from the user's supply panel to the appropriate component.

The mains for these two circuits is supplied by the user in accordance with the site requirements guide supplied.

Switching-on the Rotary pump

This circuit supplies mains for the rotary pump.

Before following this procedure ensure that the rocker switch on the side of the rotary pump is in the off position.



Warning: Check that the voltage indicated on the side of the rotary pump is the same as the supply voltage. Should the voltage need to be changed, refer to the manufacturer's manual for details of the procedure.

Close the roughing line isolation valve mounted on the rear of the IsoPrime.

Connect the IEC cable to the socket on the side of the rotary pump and connect the other end to the mains supply.

Move the rocker switch on the side of the rotary pump to the ON position.

The rotary pump should start to operate.

Switching-on the IsoPrime

This circuit supplies the mains to the electronics units within the IsoPrime cubicle (described in the 'Electrical and Electronics' section of this manual).

Ensure that the electronics circuit mains cable (from the site service panel or transformer) is connected to the inlet on the rear of the IsoPrime cubicle.

Ensure that the mains supply to the cable is switched-on.

The fans within the cubicle should begin to operate.

Switching on the EA

The EA has its own mains supply (please refer to the Site Guide for details)



Caution: Ensure that Helium gas is flowing through the EA prior to switching the EA on

- Connect the EA mains cable to the electrical supply.
- Switch on the mains supply.
- Switch on the EA by following the manufacturer's instructions.

Switching off the Electrical Supplies

Switch off is generally in reverse order of switch on.

Note: Before switching off the electrical supplies, ensure that the system is in a safe state e.g. ion Source off, pumping system closed down, etc..

The procedure is fully described in the maintenance and fault, finding section of this manual, that is in the subsection entitled 'Venting the Mass spectrometer'.

Starting The IsoPrime-EA Software

The computer system is shipped with the instrument and has the software pre-loaded, there should therefore be no need to load the software onto the system, however the software comes with full instructions on installation (any updates of the software will also come with full instructions).

This section of the manual will therefore assume that the software is already loaded onto the computer, the computer is unpacked and the various parts of the computer system (monitor, base unit and printer) are connected together according to the manufacturers instructions (for further information refer to the manufacturers guide).

Starting Up The System Software

The following procedure should be followed:

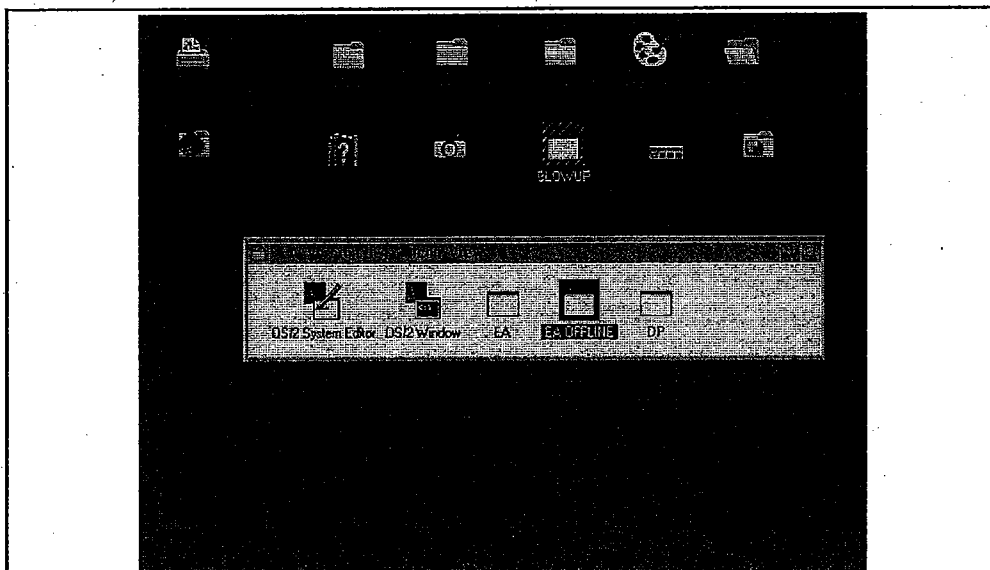
1. Connect the computer interface cable provided between the back of the IsoPrime cubicle (below the mains input) and the Computer 'Serial 1' input.

2. Connect the mains cables of the data system to the mains supply.

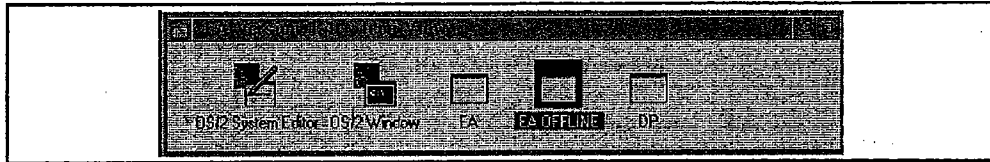


Warning: Ensure that the data system is configured for the appropriate supply voltage. See the manufacturer's instructions for details of required configuration.

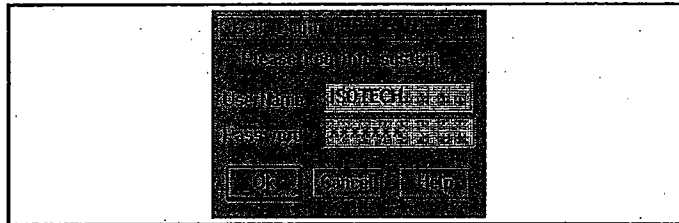
3. Turn on the computer, monitor and printer mains switches, this will bring up the OS/2 Desk Top.



4. Select EA icon and double click, this will open the EA folder.



5. Select the EA icon from the folder, a double click on the icon will start the software and the User Login dialogue box will appear.



Input the user name and password in the appropriate entry fields of the User Login dialogue box, and click on the 'OK' push button.

The software appropriate to the user level can now be accessed and the user has control of the instrument.

Notes:

The default 'Username' is MICROMASS and the default 'Password' is MICROMASS, which give the user supervisor level operation.



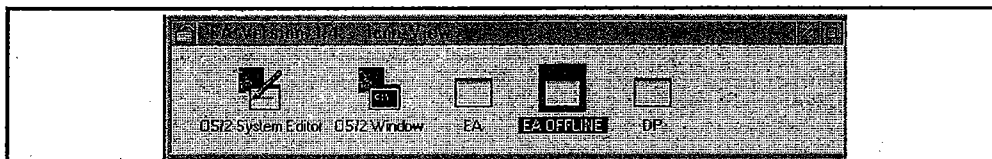
Caution: If the data system is to be accessed solely by selected users, please remove both the default "Username" and "Password" as soon as a new 'Username' and 'Password' have been set up.

- a) Refer to the fault finding section if the software fails to start.
- b) If the software has already been run and the software has been shut down with the EA folder open, then the software will re-open with this folder active and the user can start at step 5 above.
- c) If the EA icon has been placed in the 'Start-up' folder then the software will start-up at step 6 above.

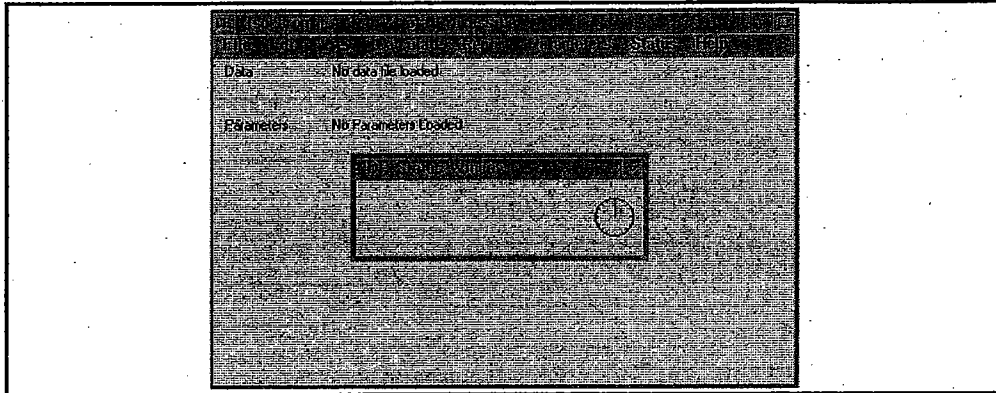
Starting Up The Data Processing (DP) Software

The data can be re-analysed using the DP software, however if the system is running an 'Autorun' where the DP software is being run, then the DP software should not be selected for manual data processing. The procedure for starting the DP software is as follows:

1. Select the EA folder (see above for details of getting to the EA folder).



2. Select the DP icon from the folder, a double click on the icon will start the software and open the DP window.



3. Select 'File' from the menu bar and load the data required using the method described in the User Interface section.

The Data can now be re-analysed using the methods discussed later in this section of the manual.

Logging In

The procedure for logging

g into the IsoPrime-EA software, if the software has been just started or the software has been logged out of, is:

1. Select 'Log In' from the User menu.

Note: If the software has just been started then ignore step 1, because the software goes directly to the Login dialogue box.

2. Enter the user name and password in the Login dialogue box. The password will not be displayed on screen.
3. Click on OK.

The user is now logged into the software and has access to the functions appropriate to the user level.

Logging Out

To log out of the software, select 'Log Out' from the User menu.

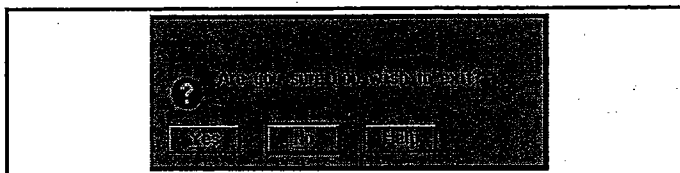
The user is now logged out of the software. However, the software remains running, which means that an 'Autorun' can be started, and then logout to prevent unauthorised interruptions or access (to regain access the user will need to log in to the system again).

Configuring the system

The system can be configured for different types of gauges and where they appear on the main window. This is done normally only once at the time of installation, however if details are required, for example, if the user is adding extra preparation system, refer to the previous User Interface section of this manual under the Config menu option.

Closing Down The Software

To close the IsoPrime-EA software down, select the system menu from the main menu bar. Selecting the 'Close' Option opens the dialogue box shown below.



Choose the 'Yes' push button to shut the program down. This takes the user back to the OS/2 Desk Top screen with the EA folder open, from which the EA or the DP software can be re-started.

Note: The system controller maintains the mass spectrometer in a safe condition even though the software is inactive (the system will remain in the same state e.g. source on, valves open, etc.).

It should not normally be necessary to exit the program, as the multitasking features of the operating system remove the need to close the software down to perform other activities. However it is necessary to close down the software if the user wishes to shut down the system controller or re-read the set-up files.

Selecting the 'No' push button will ignore the close command and the software will return to normal operation.

Pumping The Mass Spectrometer

This section of the manual guides the user through the steps necessary to achieve a vacuum in the mass spectrometer. It assumes that the system is powered up. (See the section Switching the Electrical Circuits).

There is one turbomolecular pump used on the IsoPrime and one rotary pump.

To start the Analyser Rotary Pump

1. The rotary pump should be isolated from the system using the manual isolation valve mounted at the rear of the IsoPrime prior to starting the rotary pump to avoid oil vapours, which can be produced on start up, entering the high vacuum system.
2. Ensure the rotary pump power lead is connected to the rotary pump and the mains supply.
3. Switch ON the rotary pump using the rocker switch located on the side of the rotary pump.
4. Follow the start up procedure in the rotary pump manual. It is advisable to follow the gas ballast procedure to de-contaminate the pump oil and the foreline trap sieve (if the pump has not recently been used or if the oil or sieve is new).
5. When the rotary pump has been gas ballasted, set the pump to 'high vacuum' mode and gas ballast position '0' (see manufacturers guide for details), and then leave for one hour.

Starting the turbomolecular pump

When the analyser rotary pump has been running for one hour up to the isolation valve the turbomolecular pump can be started using the following procedure:

1. Select the 'Mass Spec' menu on the data system software, Select the menu option 'Turbo on'. This will turn the turbomolecular pump on. The action of powering up the turbomolecular pump will automatically close the vent valve and the pump starts to operate.
2. Immediately after step 1, open the backing line isolation valve, located on the rear of the IsoPrime cubicle. The turbomolecular pump will then accelerate to full operating speed.
3. The Pirani pressure reading (visible in the monitor window of the software) should now start to decrease. If there are no vacuum problems the pressure will continue to fall till a pressure in the 10^{-3} mbar range is achieved.
4. When the pump has reached 95% of its operating speed, the LED on the turbomolecular pump controller will light. When this occurs the AIM gauge is enabled and the source safety interlock removed. The speed of the turbomolecular pump can also be seen in the monitor window (see User Interface section).

Notes:

1. For vacuum problems refer to fault finding section of this manual or to the relevant pump manufacturers manual.
2. It may take some time for the Pirani gauge to reach its lowest value. This depends on the amount of moisture within the analyser and the backing line. When little moisture is present it may reach this value within half an hour, however if the analyser was vented on a very humid day and no precautions were taken (see the maintenance section of this manual) this may take a full day.

The vacuum gauges

The Pirani gauge is used to measure the pressure in the mass spectrometer backing line, with the pressure being displayed in the monitor window.

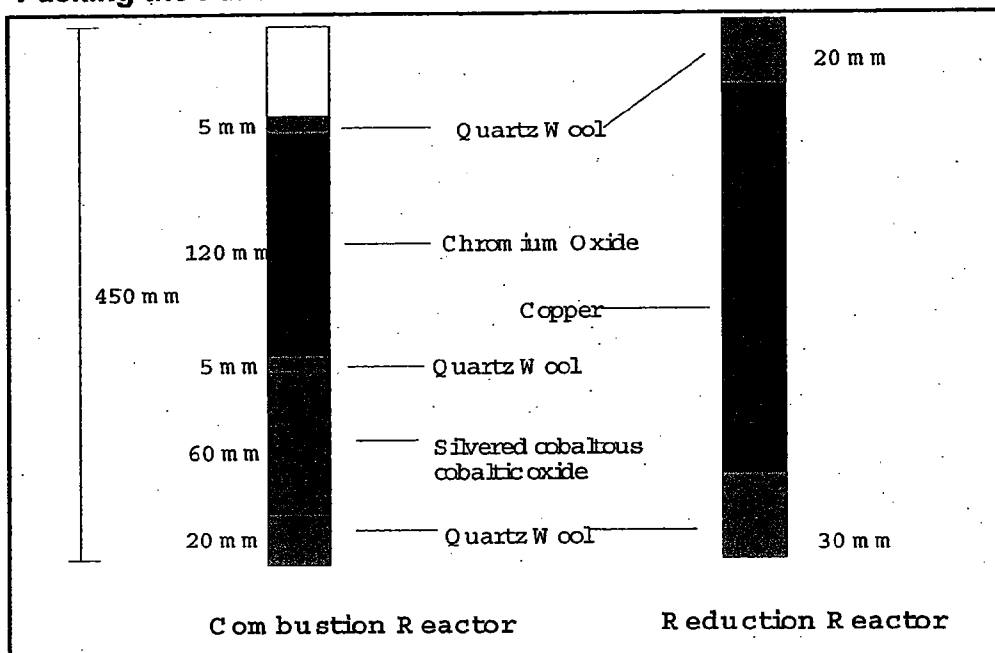
The AIM (Penning) gauge measures the pressure within the source housing. This value may be read from the monitor window. If there are no vacuum problems the pressure will continue to decrease steadily down to the low 10^{-8} mbar. It is not necessary to wait until this base pressure is reached before gas is admitted into the analyser. Gas may be admitted as soon as the pressure falls below $1\text{E-}6$ mbar.

Prep System Operations

NA2500 Set-up

The NA2500 should be set up in accordance with the instructions contained in it's user manual. Filling of combustion tubes, reduction tubes, water traps, the setting of flow rates for helium and oxygen, the furnace tube temperatures etc. etc. are all carried out according to the manual. In essence, the furnaces are packed as shown below:

Packing the Furnace Tubes



Setting the gas flows

The following settings can be used as a starting point but may be adjusted if a different column is substituted for that supplied by the factory or if a diluter is fitted.

Connect compressed air, oxygen and helium to the rear of the EA.

Set the pressure regulators on the cylinders as follows:

| | |
|-----------------|-------|
| He | 4 bar |
| N ₂ | 4 bar |
| CO ₂ | 4 bar |
| O ₂ | 2 bar |

Set the compressed air regulator on the front of the EA to a pressure of 50 p.s.i. (350 kpa).

Connect a bubble flow meter to Vent-R on the front of the EA and adjust the Reference helium regulator (R) until a flow of 40 ml/minute is obtained.

Connect the flow meter to Vent-M (M = measured, i.e. sample) and set a flow of 80 ml/min with the M regulator.

Set the pressure on the oxygen regulator to equal that just set on M. Connect a flow meter to Vent-O2 and set a flow of 25 ml/min with valve mounted next to the water trap in the middle compartment of the instrument. Leave the cap off the oxygen vent.

Set the knob below the oxygen regulator to FLOW. (STBY is used a night when the instrument is not in use to reduce the sample helium flow to a trickle and to turn off the oxygen and reference helium supplies.)

The knob under the compressed air regulator is the purge for the autosampler. Set it to ON.

Time and Temperature Set-up

These are the recommended time and temperature set-ups for nitrogen and carbon analysis, the oven temperature can be increased to, say 55°C, reduce the separation of the nitrogen and carbon dioxide peaks and hence the run time.

Set the following temperatures on the EA:

| | |
|---------------------------|---|
| Left (oxidation furnace) | 1030 °C (note the dial is multiplied by 10) |
| Right (reduction furnace) | 650 °C (note the dial is multiplied by 10) |
| Oven | 45 °C |
| Filament(TCD) | 190°C |

Set the following times on the EA:

| | |
|--------------|---|
| Cycle | 100 seconds (note the dial is multiplied by 10) |
| Sample start | 5 seconds** |
| Sample stop | 40 seconds |
| Oxygen stop | 60 seconds |
| Peak enable | 10 seconds (not used) |

This will be changed once the sample drop time has been established.

Setting the Sample Drop Time

Oxygen takes about 20 to 25 seconds to leave the loop and reach the oxidation furnace. Ideally, the sample should be dropped into the furnace 2 seconds before the oxygen arrives. A blank tin sample cup is used to find the time that the oxygen takes to reach the furnace, as it will only burn in its presence.

Initially, the drop time has been set to 5 seconds (Sample Start). Roll up an empty sample cup and place it in the sample drop position on the autosampler.

Press Start on the EA and be ready to press and hold "Memory" which will freeze the timer on the front of the EA. When Start is pressed oxygen leaves the loop. Look through the viewport under the autosampler. After 5 seconds the trap door will open and the sample cup will drop. When the oxygen reaches the furnace and the tin burns, the background in the viewport will change from orange to white. Press and hold Memory.

Take 2 seconds from the time displayed and enter it into the sample Start time.

Press stop.

Recheck the sample drop time until it is satisfactory.

All that remains is to ensure that the IsoPrime EA system software is able to

1. Read the TCD output signal from the NA2500.
2. Start and stop the NA2500 analytical cycle.

TCD output signal

Note. Columns must be at temperature and the TCD filament switched on.

ENSURE

0 - 10V signal out is used on the signal out connector of the EA.

GAIN BUTTON on the front panel of the EA is set to * 10.

The signal is displayed on the monitor and is labelled TCD.

Adjust the background signal by using the zero knob on the front panel of the EA such that the background is reading about 40 mV. If necessary use the coarse adjustment.

NA2500 start / stop

Note: In order to start the NA2500, the columns must be at temperature and the TCD filament switched on.

Make sure the helium purge supply to the carousel is in the ON position.

The EA start/stop operations are controlled by the mnemonics J5 and J4 on the IsoPrime EA prep system graphic. These simulate the manual equivalents in NA2500 stand-alone operation. The full sequence is as follows.

| SYSTEM OPERATION | EA EQUIVALENT |
|---|---|
| Double Click J5 OPEN i.e. START | PUSH EA START BUTTON |
| Double Click J5 CLOSE <i>EA green active light ON</i> | REMOVE FINGER FROM START BUTTON <i>NA2500 analytical cycle should commence.</i> |
| Double Click J4 OPEN i.e. STOP | PUSH EA STOP BUTTON |
| Double Click J4 CLOSE <i>Green active light switches to red i.e. OFF</i> | REMOVE FINGER FROM STOP BUTTON <i>The NA2500 will now complete a cycle as if a real sample was dropped</i> |

Setting Up the Reference Gas Injector

Power to the reference gas injector is automatically supplied when the instrument power is switched on. For operation of the reference gas injector, it is necessary to have connected the supplies of Helium and reference gasses

Single injector

The procedure for setting the single injector is as follows:

1. Set the supply Helium and reference gas pressures to 4 Bar.
2. Set the helium pressure to 1.5 psi on the pressure regulator on the reference gas injector module.
3. Set the reference gas pressure to 5 psi on the pressure regulator on the reference gas injector module.
4. At the reference gas vent, open the BMCV-1 needle valve until a strong flow of reference gas is observed. Let the reference gas purge for a period of 2 - 3 minutes to clear residual traces of atmospheric contamination.
5. Then reduce the flow of the reference gas to approximately 2 ml min⁻¹. This flow is measured by connecting a flow meter (bubble or electronic flow meter) to the vent outlet of the reference gas.
6. Check that the reference gas valve can be opened and closed by double clicking on the RG valve mnemonic on the inlet mimic diagram of the IsoPrime-EA software.

Notes:

- When the valve is open the plunger head of the valve protrudes a few millimetres from the valve head face. When the valve is closed then the plunger head is approximately level with the head face.
- If it is not possible to open or close the valve then please refer to the Fault Finding section of this manual.

Dual injector

The procedure for setting the dual injector is as follows:

1. Set the supply Helium and both reference gases pressures to 4 Bar.
2. Set the helium pressure to 5 psi on the pressure regulator on the reference gas injector module.
3. Set both the CO₂ and N₂ reference gas pressures to 10 psi on the reference gas injector module.
4. At the two reference gas vents, open the BMCV-1 needle valves until strong flows of reference gas are observed. Let the reference gases purge for a period of 2 - 3 minutes to clear residual traces of atmospheric contamination.
5. Then reduce the flows of both the reference gases to approximately 5 ml min⁻¹. These flows are measured by connecting a flow meter (bubble or electronic flow meter) to the vent outlets of the reference gases.
6. Check that the reference gas valves can be opened and closed by double clicking on the RG and RN valve mnemonics on the inlet mimic diagram of the IsoPrime-EA software in turn.

Notes:

- When a valve is open the plunger head of the valve protrudes a few millimetres from the valve head face. When a valve is closed then the plunger head is approximately level with the head face
- If it is not possible to open or close a valve then please refer to the Fault Finding section of this manual.

Setting the Mass Spectrometer

Introduction

When setting the IsoPrime mass spectrometer for the first time or after a prolonged period when the isolation valve has been kept closed, the steps involved for preparing the mass spectrometer are:

1. Admit gas into the mass spectrometer.
2. Check the analyser pressure
3. Switch the Ion source ON.
4. Coarse tune the Ion source.
5. Check the coincidence of the ion beams.
6. Check the cleanliness of the vacuum.
7. Leak check the mass spectrometer and the reference gas injector.
8. Fine tune the Ion source.
9. Check the stability of the mass spectrometer.
10. Check the linearity of the mass spectrometer

On a day to day basis, it is necessary only to fine tune the ion source and check the stability of the mass spectrometer, unless problems are encountered with the data.

Admitting gas into the mass spectrometer

Gas is admitted into the mass spectrometer by opening the Nupro isolation valve. This is a manual valve located at the inlet of mass spectrometer. It is described in section 4 of this manual.



Caution: It is strongly advised to follow the protocol described below. Failure to observe these instructions may result in damage to the instrument.

Please ensure that:

- The ion source is switched off
- The reference gas injection system is properly set up with its Helium supply turned on and that gas is flowing out of the reference open split.
- The EA sample line is connected to the splitter valve and helium is flowing.
- The reference capillary is properly connected to the tee.
- The pumping system of the mass spectrometer is fully operational and that the Penning gauge reading does not exceed 1E-6 mbar.

IsoPrime-EA User Manual

Now, follow the procedure described below:

1. Open the Nupro isolation valve
2. Initially there will be a surge of gas into the analyser, as gas trapped in the inlet capillaries is at atmospheric pressure. Monitor the pressure on the Pirani gauge and wait until it falls to $2\text{E-}2$ mbar. This should occur in 20 seconds or less.
3. Wait until the pressure shown on the Penning gauge falls below $5\text{E-}6$ mbar. If there are no major leaks along the inlet capillaries the pressure will fall quickly and settle to the operating pressure between 2 and $4\text{E-}6$ mbar. Failure to reach the operating pressure indicates major leaks. These must be cured before proceeding any further.
4. A continuous stream of Helium is now flowing smoothly into the ion source.

Switching the Ion Source ON



Caution: Ensure that the Penning gauge reading is less than $5\text{E-}6$ mbar.

To switch on the mass spectrometer source, access the Mass Spec menu in the IsoPrime-EA software. Select the **Source On** option. The source electronics will switch on.

- The instrument is now ready for tuning.

Coarse tuning the Ion Source

This section of the manual will look at tuning the IsoPrime ion source. It assumes that the mass spectrometer is to be tuned for CO_2 , however this procedure can be used for any of the other gas species. It will also assume that the source is turned ON and that gas is entering the source from the reference gas injector.

Recommended Values

Normally when an instrument is installed a set of source tuning parameters for each gas species of interest is provided for the user. However, if for any reason these are not available the following table provides a starting point, prior to optimising, for each gas species. The final optimum parameters for any individual machine may be some way from these values, there is no need to be worried if this happens.

CO_2 default source tuning parameters

| | |
|----------------------------|-------|
| Accelerating Voltage | 3.5kV |
| Extraction Voltage | 75% |
| Half Plate Differential | 0 |
| Z Plate Voltage | 0 |
| Electron Volts | 100V |
| Ion Repeller Voltage | -10V |
| Magnet Current (if fitted) | 3.5A |

Recommended Source Tuning Procedure

Note: Before the tuning procedure is started, ensure that the Reference gas valve is open.

This procedure should be followed for all gases, but in the example below, CO₂ will be the chosen gas.

This procedure assumes that the magnet is positioned correctly. If it is not then the magnet positioning procedure given in the maintenance section should be followed.

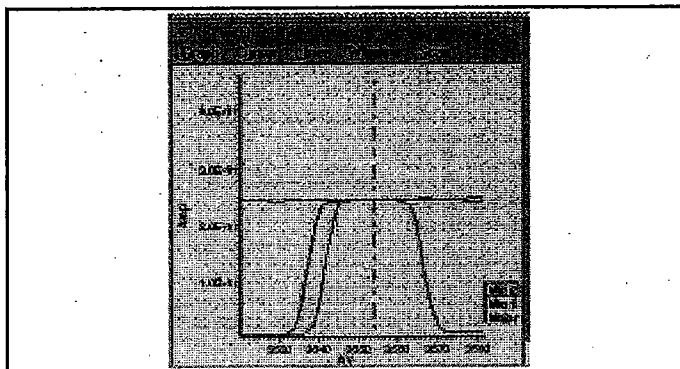
1. Load the source tuning file for the gas species to be measured, in this example CO₂. These files will have been created during the installation and have been saved under an appropriate file name. If no suitable file is available then enter the values from the table of recommended values above.
2. Adjust the accelerating voltage and/or magnet field (where an electromagnet is fitted) to focus the mass 45 in the axial collector.
3. Note: It is better to use the magnet current and only use the HT for fine adjustment.
4. Use the 'Peak Centre' command to ensure correct centering of the peak in the axial collector.
5. Run the ZV scan file and set the cursor to the maximum value.
6. Run the EX scan file and set the cursor to the maximum value.
7. Run the HP scan file and set the cursor to the maximum value.
8. Run the EV scan file and set the cursor to the maximum value.
9. Repeat the steps 6-8 until the values move by only a small amount.

Save the source tuning parameters if required.

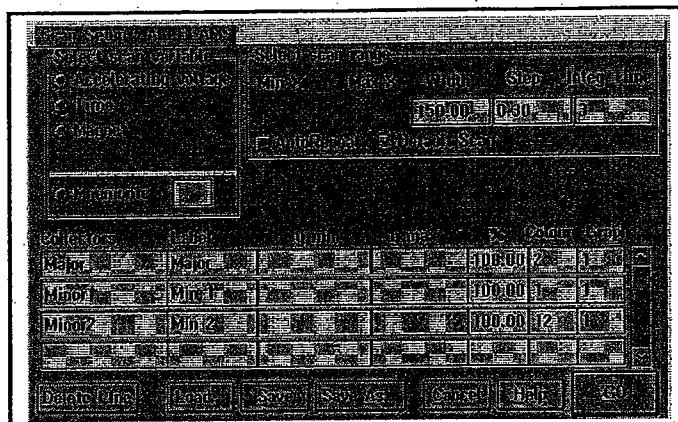
Checking the coincidence

In order to obtain good data there must be a range of HT values for which all three beams are fully focused into their respective collectors. This is indicated, as in the trace below, by the coincidence of flat tops for each of the three ion currents over the 3545V to 3559V range

If this cannot be achieved, please, refer to the maintenance section of this manual.



The coincidence trace was obtained with the scan parameters set as shown on the window below



Cleanliness of the vacuum and Leak checking

Cleanliness of the vacuum

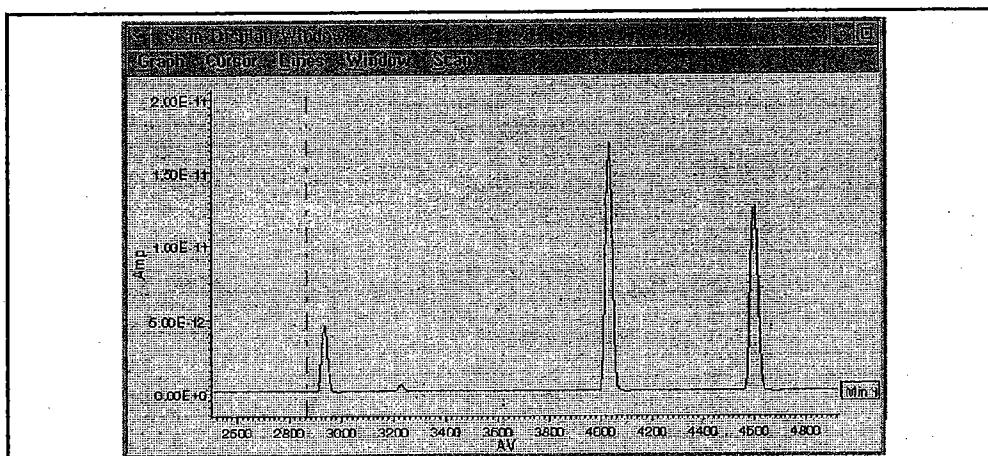
The cleanliness of the vacuum enclosure can be visualised by performing background scans.

Contamination may arise in a number of ways:

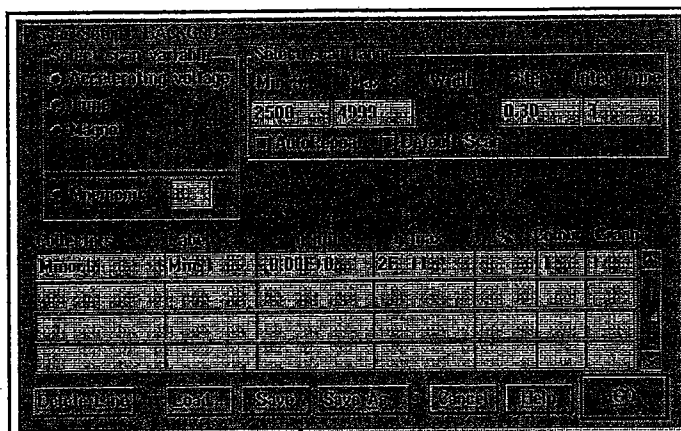
- Leak of atmospheric gases into the vacuum enclosure.
- Ingress of atmospheric gases into the carrier gas or the reference gas.
- Presence of a contaminant in a component of the system.
- Breakthrough of organic materials from the Elemental analyser.
- Backstreaming of oil from the pumps.

Whatever the source of contamination, it should be eliminated prior to running samples.

The traces below shows a typical background scan with a clean vacuum.



The scan parameters used to obtain the background trace showing the ingress of atmospheric gases are shown in the window below.



Leak checking

Severe leaks

If there are severe leaks within the interface or the elemental analyser this will become obvious by looking at the Penning gauge reading on the monitor window. The penning gauge may not switch on or may be reading a higher than normal pressure. In some cases the analyser backing line pressure may be high.

The Penning gauge is more sensitive to gases like Nitrogen and Oxygen than it is to Helium (a factor between 5 and 10). If significant quantities of air enter the vacuum enclosure then the Penning gauge will indicate a higher pressure.

Such large leaks are usually, only found on the elemental analyser. Close the Nupro isolation valve to the mass spec and leak check the elemental analyser according to the procedure described in its manual.

Minor leaks

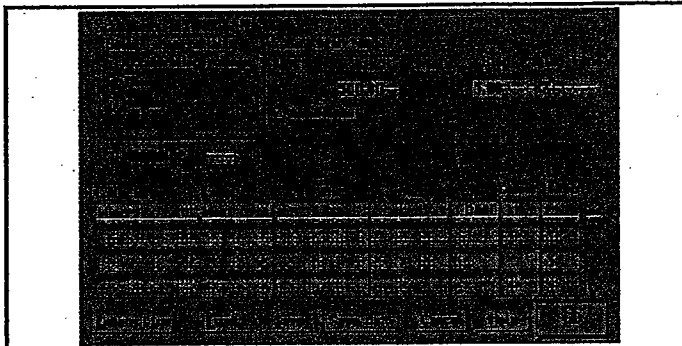
Leaks may not be sufficiently significant to cause an increase in the Penning gauge reading. These leaks will cause no damage to the mass spectrometer, they must, however, be eliminated otherwise it may not be possible to achieve the desired levels of precision and accuracy for the final sample results.

This type of leak is most easily detected using Argon gas as a leak probe.

The best method to locate leaks is to perform a 'Time Scan', with the ion source tuned to Argon, that is to mass 40.

The procedure is described below:

1. Tune the ion source to mass 45.
2. With CO₂ flowing into the analyser, do a peak centre.
3. Identify mass 45
4. Select 'peak jump' by HT, specifying the mass to jump to as mass 40
5. Start a 'Time Scan', using the scan parameters shown in the window below



Blow a gentle stream of Argon at all connections starting at the high vacuum end of the system and working your way methodically all the way back to the elemental analyser, the gas plumbing and even to the gas supply bottles. When leak checking the mass spectrometer, ensure that all possible sites are investigated. The sites include the source and collector flanges, the Nupro isolation valve and all joints of the pumping system below the bench. Look for any rise in the ion current trace on the 'Time scan' trace.

- Once a leak has been located and eliminated, continue searching for leaks through the entire system, as far as the gas supply bottles

Note: Please remember that the further away from the ion source, the longer it will take the Argon gas to travel to the ion source. For example, a leak in the reference line may cause an ion current increase within a few seconds, a leak within the elemental analyser may not cause a rise in the ion current for several minutes!

Checking the system prior to running a sample

It is assumed at this stage that:

- The mass spectrometer is fully operational and a satisfactory peak shape has been achieved.
- The EA interface is fully operational with the pressures and temperatures set and stabilised.
- The instrument has been entirely leak checked.

If the instrument has not been used for some time, the checks described in this section should be made. On a day to day basis it is necessary only to perform a stability run prior to running samples.

Backgrounds

Acceptable background levels

The table below shows maximum acceptable background levels for a number of species.

| SPECIES | MAXIMUM LEVEL |
|------------------|---------------|
| N ₂ | 5E-11 |
| Ar | 5E-12 |
| CO ₂ | 5E-11 |
| H ₂ O | 5E-10 |

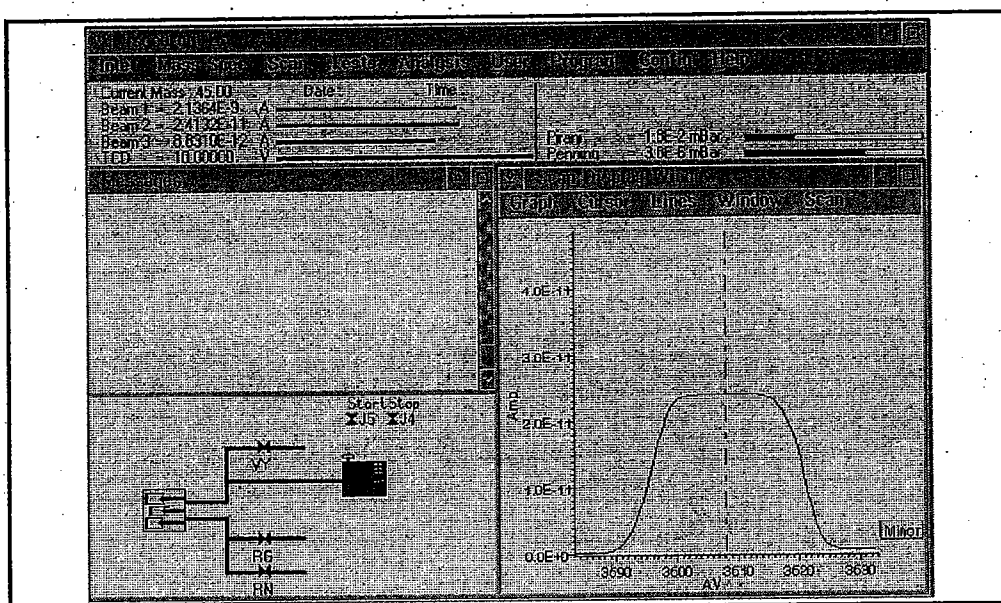
IsoPrime-EA User Manual

If the background levels exceed the values shown above, this is usually indicative of a leak from atmosphere (N_2 and Ar, contamination within the EA, possible excessive water content in the carrier gas (H_2O)). For further details, please refer to the maintenance section of this manual.

The backgrounds should be brought down to their acceptable levels or below, before attempting to run samples.

Fine tuning the Ion source

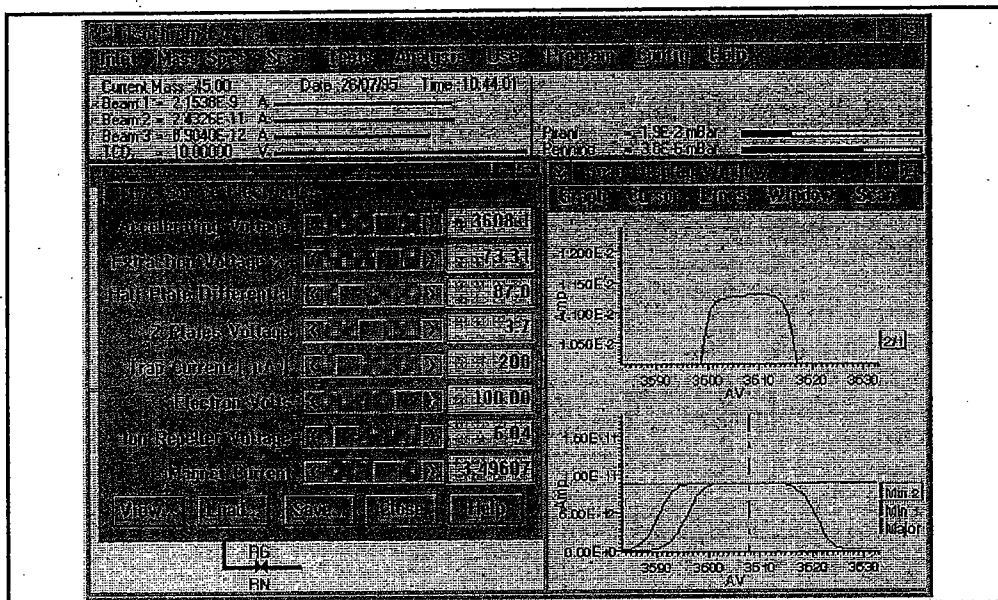
The multi tasking features of the IsoPrime-EA software are particularly useful for fine tuning the ion source. It is possible to simultaneously scan the peak shapes, tune the ion source, and observe the ion current intensities in the monitor window.



IsoPrime-EA User Manual

1. Start an auto-repeat scan, scanning the three collectors.
2. Choose 'Mass Spec', 'Tune Source'
3. Adjust the source tuning parameters using the recommended procedures, whilst observing the effect on sensitivity and peak shape.
4. When happy with the peak shapes and sensitivity, select 'Scan', 'Stop'.
5. Save the tuning if required.
6. Choose the scan command and perform a scan which includes both a scan of mass 45 in collector 2 and a scan of the 45/44 ratio.

Typical traces are shown below.



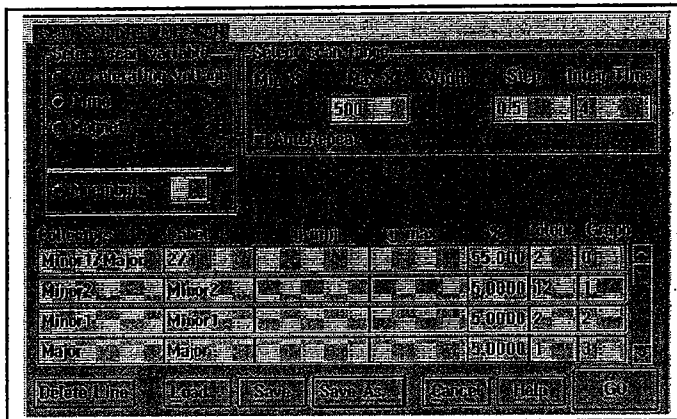
Note: If it is not possible to obtain at least 5 Volts of flat on the peak shape, please refer to the maintenance section of this manual.

Stability of the instrument

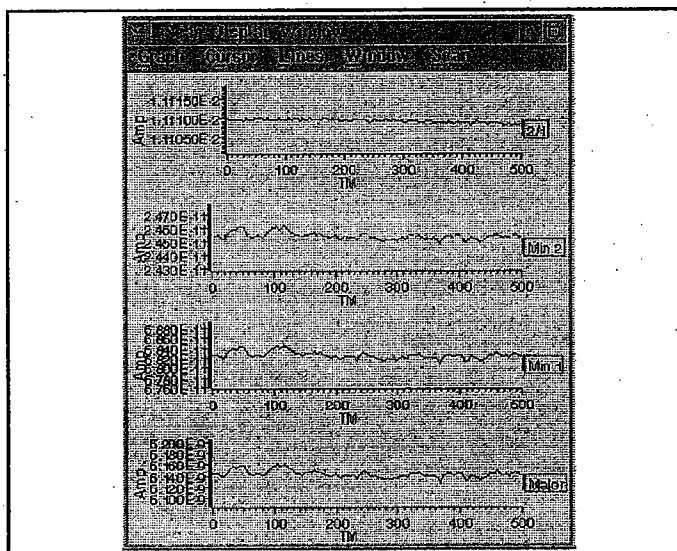
Evaluation of stability using 'Time scans'

Scans as a function of time are useful, when monitoring the stability of the system. They reveal the level of noise, the behaviour of ion currents and beam ratios as a function of time.

The parameters shown on the window below will enable the user to run a time scan for ion beams 44, 45, 46, and the ratio of beams 45/44 simultaneously, with the beams and ratio trace appearing separate graphs.



The resulting scan is shown in the figure below.

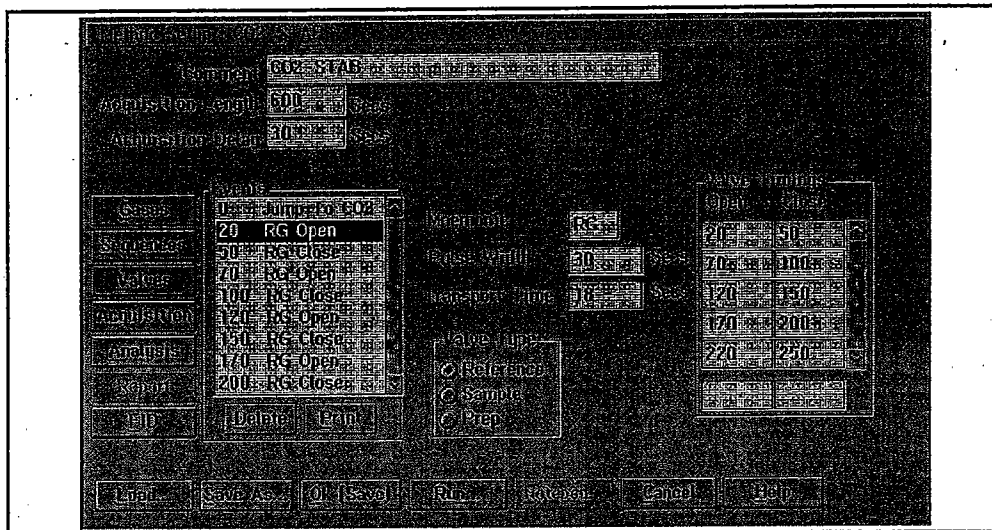


By expanding the ratio trace obtained in the 'Time scan' shown above, the average noise level for the ratio measurement can be measured. The noise in the example shown here, is contained within a band $\pm 0.00006 \times 10^{-2}$ wide. This means that the maximum precision of measurement for CO_2 that may be expected from the system is therefore around $\pm 0.005\%$.

The Reference Gas Stability Test

In order to help the user test the stability of the mass spectrometer a method has been pre-programmed and saved in a method file entitled 'CO2-Stab'. This method consists of 10 consecutive pulses of reference gas with 20 seconds between each pulse, to allow the ion beams to decay to baseline between each pulse.

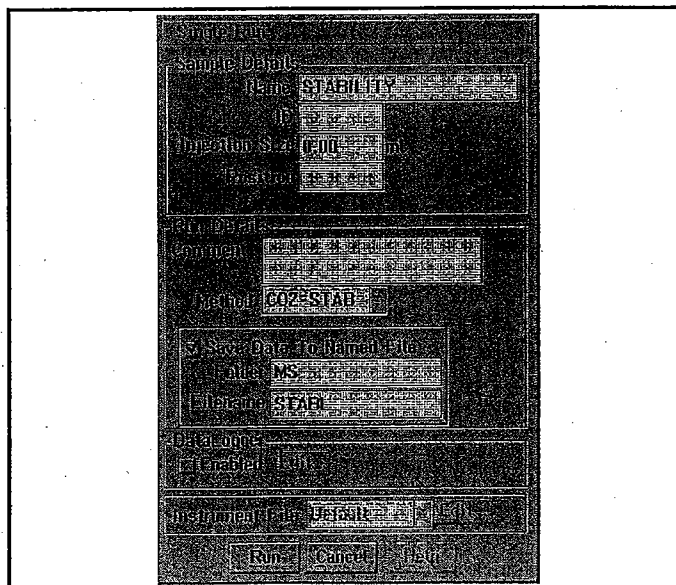
The valve timings, programmed on the valve overlay of the 'CO2-Stab' method are shown in the window below.



To run the stability test:

1. Select 'Analysis'.
2. Select 'Single run'.

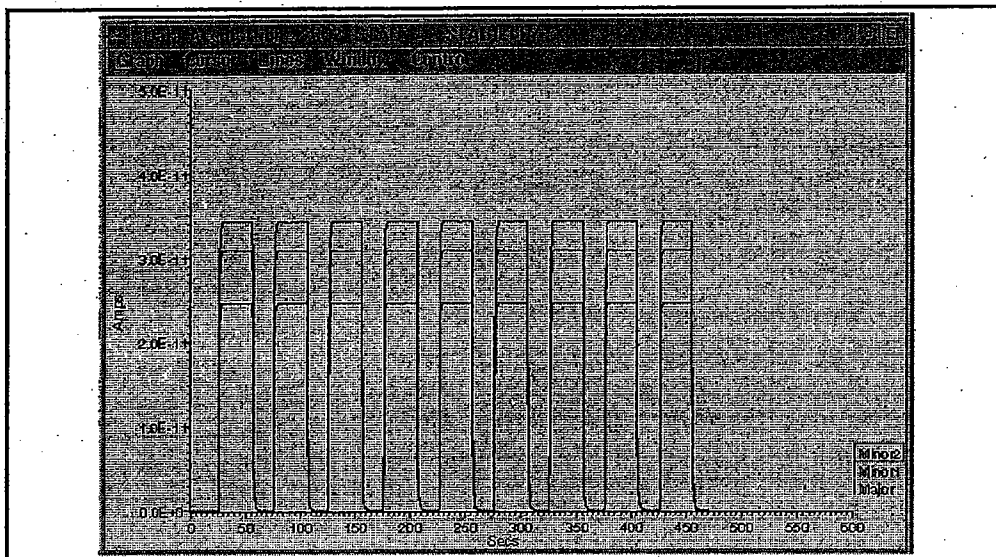
The Single Run window appears.



IsoPrime-EA User Manual

3. Select the method 'CO2-STAB', All other details are optional in this case.

Running an acquisition using this method yields the trace shown below.



IsoPrime-EA User Manual

The final report for this acquisition is printed below.

Folder : MS
Sample Name : STABILITY
Sample Position :
Injection Size : 0.0000
Sample Type : Sam
Method : CO2-STAB
Batch Name :
RunTime User : ISOTECH
Acquisition Time : 11:57:21 Date :12/04/95
Current Time : 12:08:00 Date :12/04/95

Analysis Of Reference Gas Data

Ref Delta 13 = -36.3

Ref Delta 18 = -18.7

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|----------------|----------|-----------|-----------|
| 42.3 | 1.127E-7 | 1.1104E-2 | 3.9813E-3 |
| 92.3 | 1.126E-7 | 1.1104E-2 | 3.9814E-3 |
| 142.2 | 1.127E-7 | 1.1104E-2 | 3.9814E-3 |
| 192.3 | 1.126E-7 | 1.1103E-2 | 3.9813E-3 |
| 242.3 | 1.126E-7 | 1.1104E-2 | 3.9816E-3 |
| 292.3 | 1.125E-7 | 1.1104E-2 | 3.9818E-3 |
| 342.3 | 1.128E-7 | 1.1104E-2 | 3.9818E-3 |
| 392.3 | 1.126E-7 | 1.1104E-2 | 3.9819E-3 |
| 442.3 | 1.129E-7 | 1.1104E-2 | 3.9818E-3 |
| 492.3 | 1.127E-7 | 1.1104E-2 | 3.9817E-3 |
| Std Dev Of Fit | | 1.4399E-7 | 1.2923E-7 |

Stability test report

The quantification of the stability of the mass spectrometer is indicated by the 'Standard Deviation of the Fit'

The Standard deviation of the fit in this example is 1.4399E-7

A straight line is fitted to the ten 45/44 ratios obtained from the ten consecutive pulses of reference gas by the method of linear least squares. The standard deviation of the standard errors of the points from this best fit line is calculated and reported.

A system is declared stable if the standard deviation of the fit is less than 1E-6

If this level of stability cannot be achieved, please refer to the maintenance section of this manual.

Linearity

It is essential that, whatever the ion current size within the normal measuring range of the instrument, the ion optics behave with linear characteristics.

The measuring dynamic range, defined in the specifications for this instrument, is for ion currents between $1\text{E-}9\text{ A}$ and $1\text{E-}8\text{ A}$.

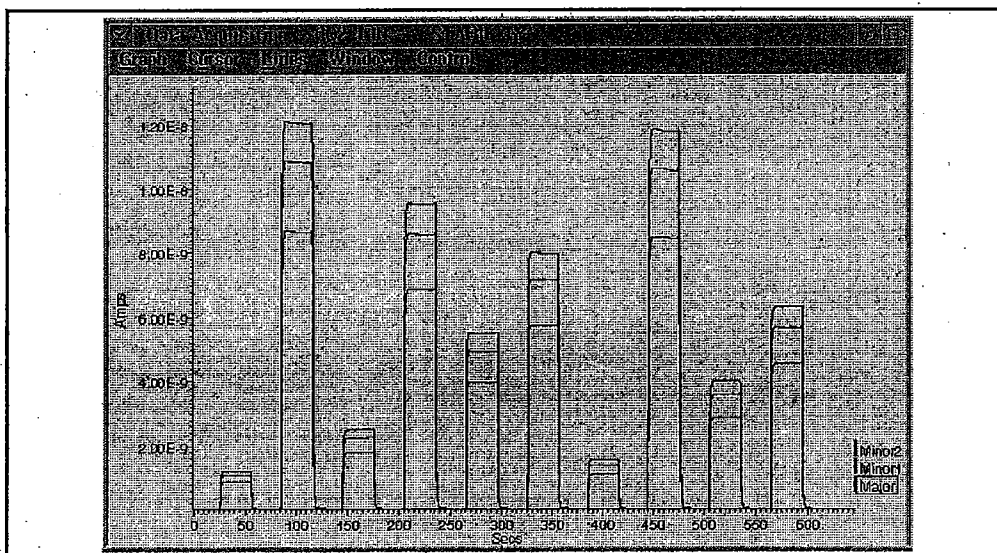
Peaks eluting from the EA column have widely different heights. In order to be sure of the accuracy of measurement for a particular peak, then it must be checked that this peak has the same Delta value, whatever its height within the allowed dynamic range.

In order to help the user test the linear characteristics of the ion optics, a method has been programmed and saved in the list of methods under the name 'CO2-LIN'.

This method consists of ten consecutive pulses of reference gas spaced by 30 seconds. Whilst acquiring data using this method, it will be necessary to increase and decrease the reference gas pressure to vary the size of the ion currents for these pulses.

The procedure, for testing the characteristics of the ion optics, is described below:

1. Start an acquisition using the 'CO2-LIN' method.
2. Vary the reference gas pressure to yield pulses ranging between $1\text{E-}9\text{ A}$ and $1\text{E-}8\text{ A}$.
3. Avoid using peak heights that systematically increase or decrease. Instead use peak heights which vary randomly across the entire range. This is shown in the window below.



IsoPrime-EA User Manual

4. Examine the final output for this acquisition.

```

Data File Name      : LINEA000
Folder              : MS
Sample Name         : STABILITY
Sample Position     :
Injection Size      : 0.0000
Sample Type         : Sam
Method              : CO2-LIN
Batch Name          :
RunTime User        : ISOTECH
Acquisition Time    : 12:25:58   Date :12/04/95
Current Time        : 12:37:26   Date :12/04/95
    
```

Analysis Of Reference Gas Data

Ref Delta 13 = -36.3

Ref Delta 18 = -18.7

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|----------------|----------|-----------|-----------|
| 42.4 | 1.971E-8 | 1.1107E-2 | 3.9863E-3 |
| 102.3 | 1.978E-7 | 1.1115E-2 | 3.9856E-3 |
| 162.3 | 4.106E-8 | 1.1111E-2 | 3.9865E-3 |
| 222.3 | 1.567E-7 | 1.1114E-2 | 3.9853E-3 |
| 282.3 | 8.998E-8 | 1.1111E-2 | 3.9856E-3 |
| 342.3 | 1.310E-7 | 1.1112E-2 | 3.9851E-3 |
| 402.3 | 2.517E-8 | 1.1110E-2 | 3.9863E-3 |
| 462.3 | 1.935E-7 | 1.1116E-2 | 3.9856E-3 |
| 522.3 | 6.539E-8 | 1.1112E-2 | 3.9861E-3 |
| 582.3 | 1.037E-7 | 1.1111E-2 | 3.9853E-3 |
| Std Dev Of Fit | | 2.6175E-6 | 5.0956E-7 |

5. Plot a graph of the ratios obtained for this acquisition as a function of pulse height

6. Calculate the slope of the graph.

The system is declared to be linear provided that the slope is less than $5E-7 A^{-1}$

Sample running

Successful sample running essentially depends on

- Achievement of good chromatography on the elemental analyser with particular emphasis on peak shape and peak separation.
- Associated timing parameters as specified in the Run Method File.

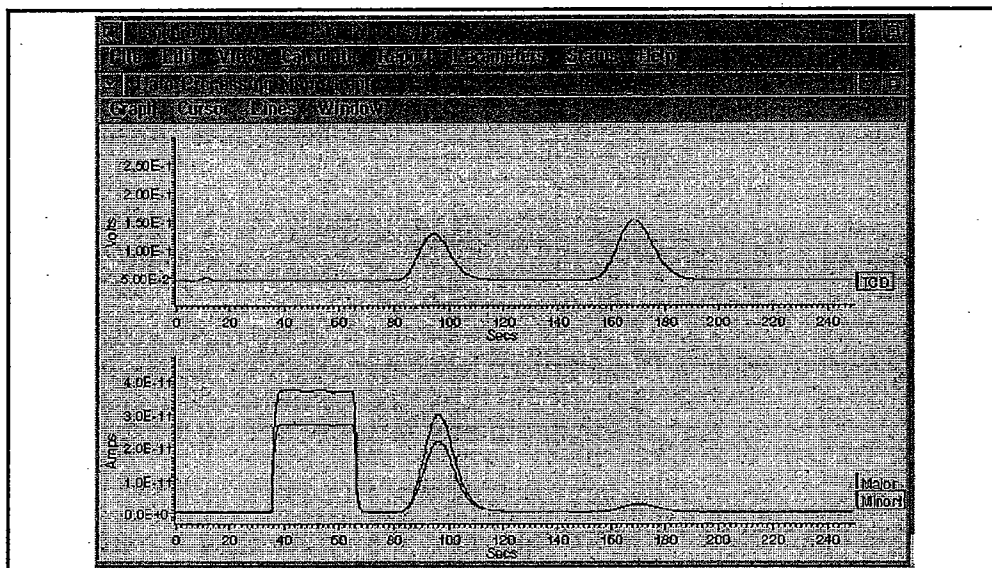
Sample chromatography

Assuming the elemental analyser is set up in accordance with the instructions given in its manual, chromatography should not be a problem. If, however problems are encountered please refer to section 8 of this manual.

Run method file timings

All of the following should be related to the associated diagram showing a typical chromatographic output for both the elemental analyser and the mass spectrometer. Three sample run file configurations namely N₂, CO₂, N & C will be discussed. Topics such as Sulphur or the use of the EA diluter will be covered as an appendix. Most of the major aspects of defining the run file will be discussed for the case of nitrogen, after which subsequent run files will only contain new information specific to each. This section must be read in conjunction with section 5, the software section.

N2 Run method file



IsoPrime-EA User Manual

The following aspects of data acquisition have to be considered.

- **TCD peak elution times**

Generally nitrogen appears at 90 - 130 seconds, Carbon at 170 - 220 seconds.

TCD zero right offset. Set to 5 seconds

TCD zero left offset. Set to 5 seconds.

TCD zero duration. Set to 3 seconds.

TCD peak threshold. Set to 0.02V above TCD background.

Access from events (TCD) in method file.

- **Left and Right zero details.**

Usually, but not always, in single species method files there will be 2 points specified in the mass spec chromatogram where the background zero values are read. Normally at positions-

Z1 Left zero Set to 10 seconds from start.

Z2 Right zero Set to 10 seconds from end.

The zero width is set to 3 seconds.

The main criteria in determining the zero positions is that they should be spaced as far apart as possible and that they represent true background.

Access from events (GAS) in method file.

- **Reference Gas pulse time.**

Only one pulse is required and is introduced via the reference line prior to the arrival of the sample peak at the mass spec. For an average transport time of 12-20 seconds the reference gas valve RN is instructed to open at 20 seconds and close at 50 seconds giving a width of 30 seconds. Try to ensure that the reference pulse does not get too close to the nitrogen peak (the reference pulse should end 10 seconds or more before the nitrogen peak rises).

| RN Open | RN Closed |
|---------|-----------|
| 15 | 45 |

IsoPrime-EA User Manual

These times are entered from the events (VALVES).

The width of the reference gas pulse is user defined. It is declared on the RN valve time overlay of the Method window.

Note: The default width of the reference gas pulse is 30 seconds. This time was determined experimentally to be an optimum width. The precision of measurement may be affected if less than 30 seconds are used, it does not, however, improve for pulses longer than 30 seconds.

On 'tabbing out' of the 'RN open' field, the 'RN close' time is automatically entered by the software which takes into account the pulse width declared. The cursor then positions itself on the next line, ready to accept the next 'RN open' timing.

Transport Times for RN

This is the time that it takes for the gas to flow from the RN valve, all the way to the ion source.

To measure this time:

1. Start a Time scan and open RN at 20 seconds - let this be T_1 (seconds).
2. Wait for the pulse to elute on the ion beam trace.
3. Use the 'Raw Data' to measure the time at which the pulse starts to appear on the trace. Let this be T_2 (seconds).
4. Then the transport time is $T_2 - T_1$ (seconds).

This is the time that it takes for a reference pulse to travel from reference gas valve to the ion source.

● Defining the sample window

The sample window is defined to be that segment of the mass spec chromatogram that wholly includes the sample nitrogen peak eluting from the elemental analyser from prior to its arrival to after it has completely decayed. It is specified using the HS valve timings normally associated with the IsoPrime GC. Although this valve does not exist in the IsoPrime EA hardware, declaring its actions will define the sample window.

| | | | |
|----|--------|--------------------|-------------------------|
| HS | CLOSED | Set to 75 seconds | Start the sample window |
| HS | OPEN | Set to 140 seconds | End the sample window |

HS Valve timings

| OPEN | CLOSED |
|------|--------|
| 140 | 75 |

Times are entered from the events (VALVES)

IsoPrime-EA User Manual

Note: In the example chromatogram above there is a complication to the detection of the nitrogen peak due to the presence of CO₂ from carbon in the sample. As the CO₂ enters the mass spec 7 - 10 % of it will crack in the source to CO, which will of course be detected, like nitrogen at mass 28. The result is 2 peaks appearing on the mass spec chromatogram. HS OPEN must be set before the CO peak appears regardless of whether the Nitrogen peak has reached baseline. This complication can be removed by trapping the CO₂ out according to the directions in the elemental analyser manual. Alternatively arrange the elemental analyser conditions to give as much chromatographic separation as possible.

- Defining the sample height

To qualify as a real sample peak the peak must satisfy user defined conditions on the peak to baseline height and the peak to zero height. See section 5 for more details. Typical values are;

Peak to baseline 5E-11A

Peak to zero 6E-11A

Note: Peak to zero is usually larger than Peak to baseline providing the zero positions are specified correctly.

Conditions are set from the Analysis window overlay.

- Acquisition delay

The acquisition delay is the time it takes for the ion currents to return to their background levels after RN closes. On starting a run, RN closes automatically. The data acquisition will not start until the declared acquisition delay time has elapsed. To measure this time, please follow the procedure outlined below:

1. Open ref gas valve RN. Ion beams rise to maximum.
2. Wait 2 minutes.
3. Close RN, make a note of the time. = T₁
4. Wait till ion beams decay to a steady background value.

Make a note of the time. = T₂

The delay time is then T₂ - T₁

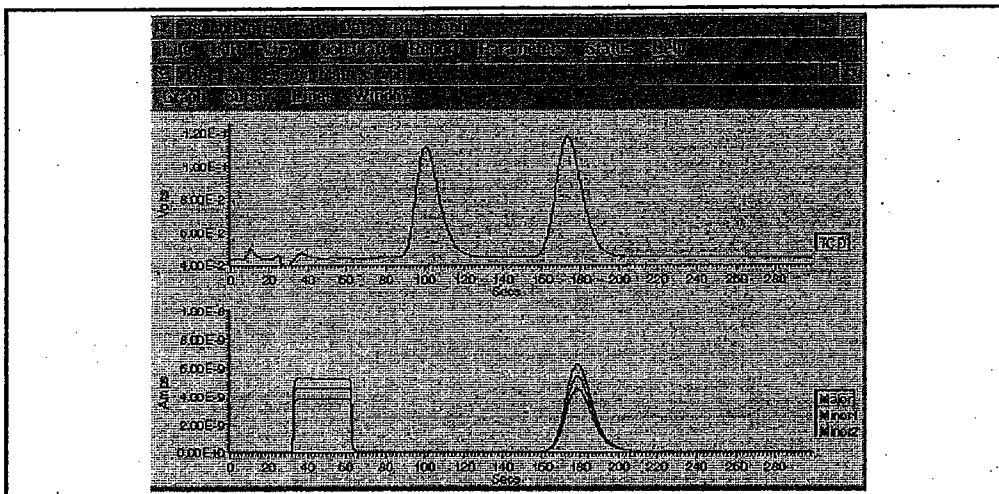
- Acquisition length

The length of the data acquisition depends on a number of variables.

1. The time taken for the sample peaks to elute from the elemental analyser.
2. Whether CO₂ is trapped in the elemental analyser.
3. Quality of the chromatography (tailing effects).

In general, an average time for Nitrogen would be about 240 seconds for an elemental analyser using a 2 metre column.

CO₂ Run method file



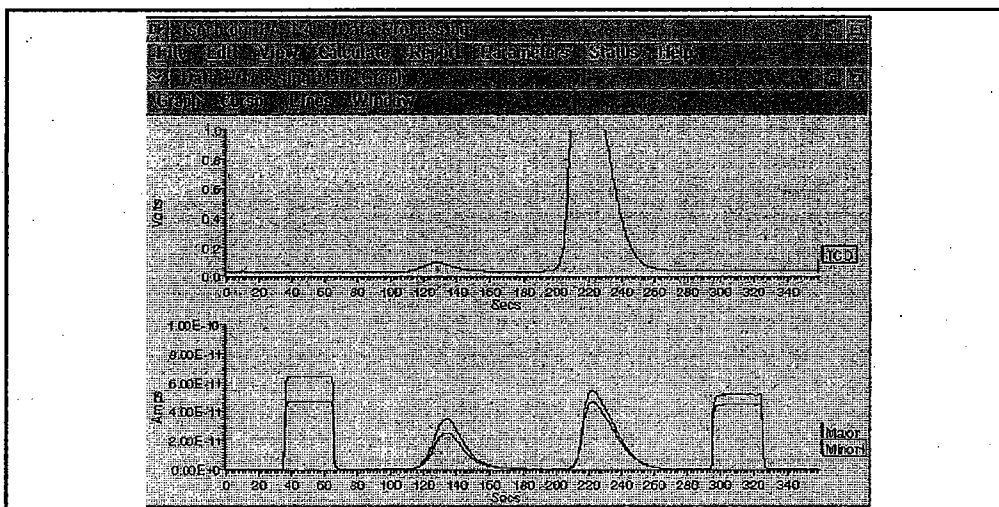
Major differences are:

1. Nitrogen does not appear on the mass spec trace, so defining the sample window is easier. Normally about 140 - 299 seconds. Specify the HS open action just before the end of the acquisition time
2. Run time is longer, normally about 300 seconds.
3. Although Nitrogen does not nominally appear on the mass spec trace, its entry to the source can affect the monitoring of CO₂ so avoid placing the CO₂ reference pulse during this time.

NC Run method file

The introduction of simultaneous acquisition of nitrogen and carbon in the same sample demands a very clear appreciation of a number of instrument and analytical characteristics.

Please refer to the example chromatogram.



The chromatogram falls easily into 3 areas.

- a) The region before the peak jump where the acquisition of Nitrogen takes place.

IsoPrime-EA User Manual

The instrument must be tuned to nitrogen. Due to time constraints associated with the peak separation it is normal to have only 1 zero position placed at the beginning of the chromatogram. The method file must include details of how the peak jump from N₂ to CO₂ and back takes place. This can be by loading tuning files, peak jumping HT or peak jumping using the magnet. The nitrogen reference valve pulse must be specified. The sample window must start before the N₂ peak appears and must end before the CO₂ reference pulse. Note the sample window encompasses both peaks.

b) The region after the peak jump where the acquisition of CO₂ takes place.

Again, due to time constraints associated with the peak separation, only 1 zero position at the end of the chromatogram will be defined. The CO₂ reference pulse must be placed after the sample window ends.

c) The peak jump itself where the transition from N₂ tuning to CO₂ tuning takes place.

The timing of the peak jump, how long to give the source to stabilise (Settle time). Defining the peak jump method (HT, tuning, magnet?) are the main considerations in this region.

Method Setup

- **Setting up peak jumping using tuning files.**

Open both reference gas valves RG and RN.

Load the nitrogen tuning file ().

Peak centre on mass 29 and save the tuning.

Load the CO₂ tuning file (CO2.TUN).

Peak centre on mass 45 and save the tuning.

Both centre voltages should be around 3.5kV Accn Volts.

Repeat this process until comfortable that switching from one tuning file to the other will result in the system being set to the centre voltage of the mass being jumped to.

Load N2.TUN again, identify mass 29, and close both reference valves.

- **Method file checklist**

Load NCRUN Method file.

Peak jump is enabled, the jump mode is set to tuning and the correct tuning files are input using the jump mode EDIT button.

Left zero position specified for N₂ in the Gas and peakjump overlay. The right field has no entry associated with it.

Right zero position specified for CO₂. The left field has no entry associated with it.

CO₂ jump time set at least 10 seconds before arrival of the CO₂ peak.

Settle time set to 6 seconds. (Allows time for the magnet to stabilise)

Sample window defined by HS starts before the nitrogen peak and ends after the CO₂ peak.

Acquisition length set appropriately.

IsoPrime-EA User Manual

- **Peak jump considerations**

Selecting peak jump using the magnet changes only the magnet current, all other source parameters remaining the same. Not normally used.

Selecting peak jump using the HT changes only the acceleration voltage, all other parameters remaining the same. Not normally used.

Both these options can be used whereby other source parameters are changed by setting up specific tuning files and enabling peak jump using source tuning.

Eg

N2X.TUN is set up as normal for nitrogen. Magnet current around 2.6 Amps, HT 3.5kV.

CO2X.TUN is set up for CO₂ at a much lower HT, around 2.6kV, but the magnet current is the same as for the N2X file. Apart from the magnet current both files are tuned independently.

If these files are incorporated into a peak jump by tuning method, then the effect will be as if peak jump using HT was enabled but with the addition of changing the other source parameters.

It is sometimes advisable to use this method of peak jumping in cases where by design or accident the peak separation is poor since any jump not involving the magnet is much faster. In this case the settle time can be reduced to 1 or 2 seconds.

Selecting peak jump using source tunings whereby both tuning files are tuned for sensitivity, both files have approximately the same HT setting at 3.5 kv and only the magnet current is very different. This is the normal method used.

- **Sample characteristic considerations**

For samples with high C:N ratios and in the absence of a diluter, special considerations have to be made in terms of how to run the sample.

In normal operation it is possible to measure up to a C:N ratio of 10:1. For ratios greater than this a method has to be found which will independently boost the nitrogen sensitivity and/or reduce the CO₂ sensitivity. One way to do this is to through source tuning manipulation. Maximise nitrogen sensitivity, save as N2X.TUN, reduce CO₂ sensitivity and save as CO2X.TUN. Use these files when setting up the method. It is possible to mix source tuning files in an autorun, but concentration measurements must then be obtained using the TCD signal from the elemental analyser.

BlankN method file

Whenever a sample is dropped into the combustion region of the elemental analyser a small but significant amount of atmospheric nitrogen is also introduced. There are two main sources of this nitrogen.

1. Air getting past the helium purging system of the carousel slides during the sample dropping action. This is the main source.
2. A small amount of nitrogen trapped inside the tin cup along with the sample.
3. An almost negligible amount associated with the tin itself.

IsoPrime-EA User Manual

The function of the N₂ blank method file is to acquire data representing the integrated beam areas produced when an empty sample cup is dropped. These integrated areas can then be used to correct the beam areas generated by a real sample.

i.e. True sample area = measured sample area - blank area

The correction is applied after all the samples and blanks have been measured.

Setting up the method file

The method file is almost identical to the N2RUN file (see above). The only additional considerations are:

Specifying peak to zero and peak to baseline to allow for the fact that the peak heights by definition will be very small.

BlankC method file

Source of the blank here is organic residues associated with the tin capsules. The Method file is almost identical to CO2RUN file apart from having to respecify the peak to zero and peak to baseline height.

BlankNC method file

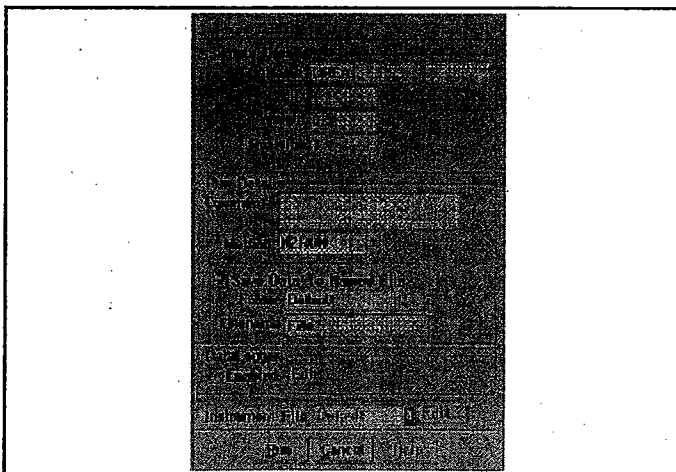
Used in correcting data associated with the analysis of nitrogen and carbon in the same sample. The method file is almost identical to the NCRUN apart from having to respecify the peak to zero and peak to baseline height.

Starting a single run

Having defined a method file in terms of timings etc it must be tested prior to incorporating it into an autorun. The best way of doing this is to run any convenient sample as a single run. By use of the manual DP option (described in the next section) the resulting chromatogram can be checked for any fine adjustments in timings required by the method file.

Selecting the single run option

1. From the main menu bar, select 'Analysis'.
2. Select 'Single Run'.



3. Please ensure that the N2RUN method is selected.

IsoPrime-EA User Manual

4. Ensure, 'save data to named file' option is ticked. In this case the folder is 'Default'
5. Press 'RUN'.

The selected method file is now run.

The final report

At the end of the data acquisition, a single run, results report will be generated.

Data Processing Results

| | | | |
|------------------|---|----------|-----------------|
| Data File Name | : | File_000 | |
| Folder | : | Default | |
| Sample Name | : | UREA | |
| Sample Position | : | 1 | |
| Sample Weight | : | 0.3200 | |
| Method | : | N2RUN | |
| Batch Name | : | | |
| Runtime User | : | ISOTECH | |
| Acquisition Time | : | 11:57:09 | Date : 26/07/95 |
| Current Time | : | 12:02:48 | Date : 26/07/95 |

Analysis Of Reference Gas Data

Ref Delta 15 = 0.00

| | | | |
|----------------|----------|-----------|-----------|
| Time | Major | Ratio 2/1 | Ratio 3/1 |
| 48.0 | 5.867E-8 | 7.1337E-3 | 1.1603E-4 |
| Std Dev of Fit | | N/A | N/A |

Analysis Of TCD Peaks With Zero Subtraction

| Time | Label | Height | Area | T(L) | T(R) | H(L) | H(R) |
|-------|----------|--------|-----------|-------|-------|----------|----------|
| 128.0 | Nitrogen | 6.6E-1 | 1.3642E+1 | 104.0 | 171.5 | 1.930E-2 | 2.000E-2 |
| 219.3 | Carbon | 6.9E-1 | 1.6496E+1 | 191.0 | 290.7 | 1.920E-2 | 2.530E-2 |

Analysis Of Sample Peaks, With Zero Subtraction

| N2 Time | Height | Area | 2/1 | 3/1 | dN15Pk | Atom % |
|---------|--------|--------|-----------|-----------|--------|--------|
| 131.0 | 1.2E-8 | 2.3E-7 | 7.1558E-3 | 1.2018E-4 | 3.10 | 0.3674 |

The following is an explanation of the content and terminology of the report

- Data file name Where the raw data is stored.
- Folder Where the data files are stored
- Sample name As specified
- Sample weight As specified
- Sample type Not used in single run mode
- Method The method file specified
- Batch name Not used in single run mode
- Runtime user The log on name
- Acquisition time The time the data was acquired
- Current time Always updated to present time

IsoPrime-EA User Manual

Analysis of reference gas data

- Ref delta 15 Isotopic value of the reference gas wrt AIR
- Time Centre of reference gas pulse
- Major Area in Amp.Seconds of Major beam
- Ratio 2/1 Minor1 beam area / Major beam area
- Ratio 3/1 Minor2 beam area / Major beam area
- Std Dev of fit Not valid unless there are 3 or more reference pulses. Normally there is only one.

Analysis of TCD peaks with Zero subtraction

- Time The time the peak appears at the TCD
- Label Gas species as defined in the method file at these times
- Height TCD peak height in volts
- Area TCD peak areas in volt. seconds
- T (L) Start of the peak in seconds
- T (R) End of the peak in seconds
- H (L) Background TCD signal to the left of the peak
- H (R) Background TCD signal to the right of the peak

Analysis of sample peaks with zero subtraction

- N2 time Time at which the peak appears on the mass spec
- Height Major beam height
- Area Major beam area
- 2/1 Sample minor1/major isotope ratio
- 3/1 Sample minor2/ major isotope ratio
- dN15Pk Sample delta wrt AIR
- Atom % Absolute atom% N15

Introduction to manual data processing

The data reprocessing facility (DP) exists in two forms.

- a) Manual DP which can be called from the desktop. This is generally used for re-examining data associated with single runs and is the topic of this section.
- b) Batch DP, which can be called from within the IsoPrime EA software. This is generally used for re-examining data associated with multiple sample autoruns and will be discussed later.

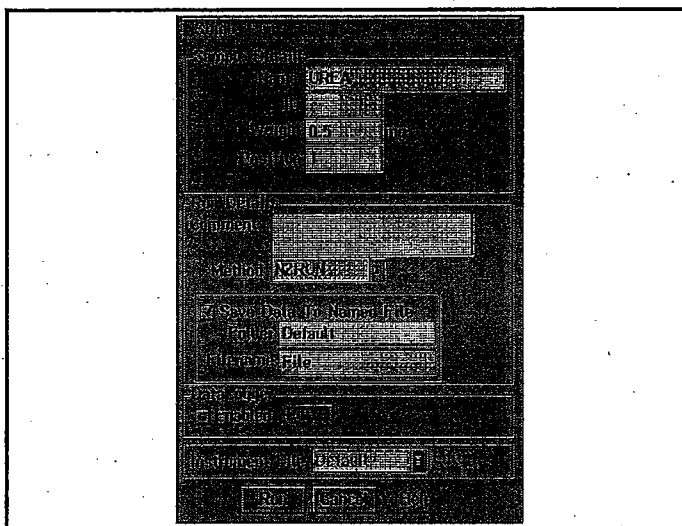
IsoPrime-EA User Manual

The purpose of the DP facility is to allow the user to access, manipulate and generally study in close detail all aspects of the data acquisition. Timing aspects of most of the parameters can be altered, and the data reanalysed to see what effect this has on the results. Many other aspects of the data analysis can similarly be treated. Please note however that it is not possible to gain access to or alter the fundamental raw data.

Accessing manual DP

Once a single run has been carried out in order to check the method run timings.

- The data reprocessing software is accessed through the *DP* icon in the *EA* folder. Double click on the *DP* icon to start the program. The *DP* window appears.



The menu bar of the *DP* Main Window facilitates loading of data, and loading and saving of parameter files. Once data and a parameter file are loaded, the remaining menus on the menu bar become active, and control various aspects of the display of information, and alteration of the reprocessing parameters.

Loading the single run data file

1. At the *DP* Main menu bar select the **File** menu and choose the **Load Data** option. The **Load Files** window appears.
2. A list of folders containing single run files will appear. Scroll to and double-click on the **Default** name in the folders list box. A list of single run data files within the default folder will appear. The most recently acquired will appear at the top of the list.
3. Either double-click on the file name, or click once on the file name to highlight it then click on the **OK** button. The **DP Main Graph** will then display both the TCD and Mass spec trace associated with this file.

Checking the method file parameters

1. At the *DP* Main graph menu bar select the **Cursor** menu choose the **Raw Data** option and check the actual times that the TCD and mass spec peaks appear on the chromatogram. Make a note of any changes that may be required in the method file setup.

IsoPrime-EA User Manual

At the DP data processing main menu select **File** and choose **Initialise parameters** option. The graphs will be redrawn and all of the greyed out options associated with this window will be activated. Select **View** and tick the **Zero** option. The DP main graph will be redrawn showing the position of the background zeros as specified in the method file. Make a note of any changes that may be required in the method file.

Use this feature of the software to check any other aspect of the sample run eg peak to baseline, peak to zero during the analysis of blanks etc. Once the method file parameter settings required are found close out of the DP software. Make changes in the method run file and check them out with another single run.

Closing the DP Software

To exit the *DP* software proceed as follows....

1. Use the **Save Params** option of the **File** menu to save any modified parameters in a .000 parameter file
2. Exit the DP software by:
 - either double-clicking on the **System Button** on the top left corner of the DP main window title bar (this is marked DP)
 - or click once on the **System Button** to reveal the **Window Control Menu**, then select the **Close** option
3. Click on the **Yes** button in the confirmation dialogue box.

Setting up an autorun

Introduction

The autorun facility of the IsoPrime EA software incorporates many powerful features designed to make the system as flexible and user friendly as possible. For example samples can be loaded onto the carousel and the details appended to the batch file while the autorun is running. This means the system can usefully be analysing all through the weighing procedures. A data logging facility allows for the export of data direct to spreadsheets.

Creating a new autorun file

Having characterised the order in which to run the samples, create an autorun batch file as follows.

| | | | |
|--------|---------------------|---------------------------------|------------------|
| Select | Analysis | | |
| Select | Autorun Edit | | |
| Select | New | Type the name of the batch | Select OK |
| Select | Procedure | | |
| then | Edit | to define data logging options. | |

IsoPrime-EA User Manual

Note: Performing data logger on Batch DP is the more useful data logging option at the moment. Performing data logging on each sample of a long autorun does work but the form of transfer to the spreadsheet is such that a great deal of manipulation is often required to make sense of it. The method of individual sample data logging is at present under review

DEFINE the sample details

Place cursor in **TYPE**. Select sample type using **Combo box**.

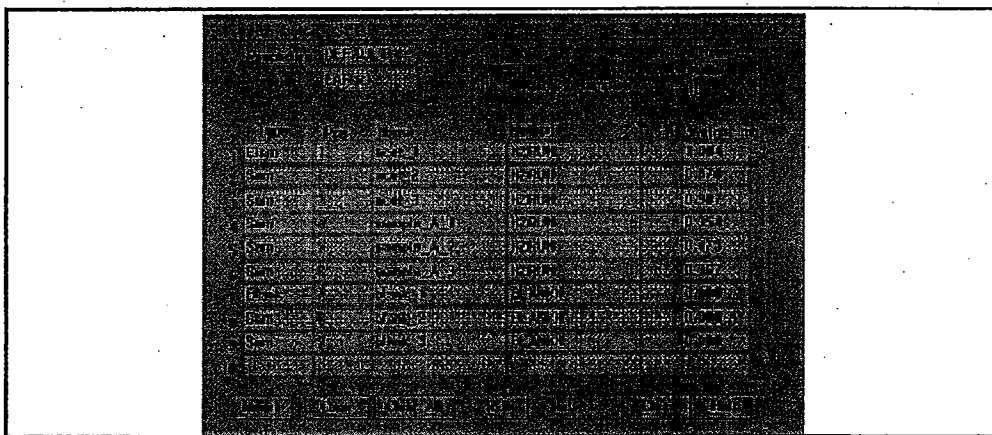
Tab to **NAME**, Insert sample title.

Tab to **METHOD**. Select method using **Combo box**.

Tab to **WEIGHT**. Insert weight in mg.

Tab to next sample

When the weight of the last sample has been entered press **RUN** to start the autorun. Do **NOT** press **RETURN** or **TAB** after entering the weight of the last sample.



Note

The first letter of the options contained in the Combo box can be used to insert the type, method, directly from the keyboard.

E.g.

In the method field type **N** to select **N2RUN**. If more than one **N** exists then repeatedly striking **N** on the keyboard will cycle through all the possibilities.

Autorun options

- **Loading up a full batch and running the autorun**

Described already in the subsection **Creating a new autorun file**

- **Updating a batch during the autorun.**

Allows for the first 6 or so samples to be loaded, the autorun to be started, and further samples to be added to the batch as the autorun proceeds. This is probably the most useful feature to the analyst as it reduces the amount of instrument idle time to a minimum. The use of this feature depends on the analytical cycle being longer than the weighing cycle. Typically 2 samples can be weighed and loaded in 5 minutes during which time only 1 sample is analysed. All that is required, as in any autorun sequence, is that the sample order running is known in advance.

Procedure

1. Weigh 6 samples. These may be blanks or reference materials or actual samples, depending on how the run is structured.
2. Load the samples on the EA carousel and set up a new autorun batch file as described in Creating a new autorun file.
3. When the weight of the last of the initial samples has been entered press **RUN**.
4. The batch file will remain at the front of the screen while the autorun begins in the background. As samples are accessed to be run, their sample details will be greyed out.
5. Weigh out more samples and load them onto the carousel. Select the next sample in the batch file by clicking on the type box with the cursor. Enter the next set of sample details.

Alternatively

After pressing **RUN** in 3 above, close out of the batch file by pressing **CLOSE**.

The system will then display the chromatogram at the front of the screen.

Weigh out the next batch of samples.

To re-enter the batch edit,

Select **Analysis**

Select **Autorun Edit**

Select **Running**

The current batch file will be loaded to which the next set of samples can be added.

Precaution

Do not allow the autorun to process the last sample in the current batch list if there are still more to add. Otherwise the current autorun will stop. If this does happen inadvertently, please see the section on **Appending to a completed run file**.

● **Appending to a completed run file**

There are situations where it makes sense to reactivate a completed autorun batch file. For example, the user may be using a batch file labelled 'testN' which is used to characterise unknown samples. The data associated with these files is not in itself important except that it allows the user to decide how best to run the sample in a 'real' run. To add samples to this file

Select **Analysis**

Select **Autorun Edit**

Select **Run**

Load the required batch file and append the sample details. Select **Run**.

IsoPrime-EA User Manual

- **Aborting the autorun**

If for any reason it is desired to halt the autorun, then

Select **Analysis**

Select **Autorun Stop**

The system will complete the present sample being analysed and then stop. If the analysis is to be stopped immediately then after selecting autorun stop,

Select **Program**

Select **Terminate**

- **Restarting an interrupted autorun**

Select **Analysis**

Select **Autorun**

Select **Interrupted**

The interrupted batch file will appear. Check that the last sample greyed out has in fact been run then press **RUN**.

Autorun batch summary report

After the final sample has been completed the batch summary report for the full autorun will be produced. A typical example of this report is shown.

Batch Data Processing Results

Data File Name : MEMSOIL1
 Autorun Setup File Name : MEMSOIL1
 Blank Subtraction : Disabled
 Background Subtraction : Enabled
 Reference Gas : Enabled
 Ref Gas Delta (C13) : -36.40 Ref Gas Delta (018) : -20.00
 Ref Gas Delta (N15) : -1.00
 Current Time : 11:04:53
 Current Date : 26/07/95

Elemental Calculations using TCD Areas

Avg Sensitivity for C (Vs/mg) : 9.226E+1
 Avg Sensitivity for N (Vs/mg) : 3.136E+1

| No. | Sample Name | Details Weight (mg) | Ref Type | Elemental (C) | % Comp (N) | Isotopic (C) | Delta (N) | |
|-----|-------------|---------------------|----------|---------------|------------|--------------|-----------|---|
| 1 | LCP3_1 | 30.3920 | Elem | 1.590 | 0.120 | | | R |
| 2 | LCP3_2 | 29.9640 | Sam | 1.605 | 0.121 | -28.98 | 9.03 | |
| 3 | LCP3_3 | 29.7640 | Sam | 1.609 | 0.129 | -28.82 | 8.91 | |
| 4 | LCP3_4 | 29.7840 | Sam | 1.585 | 0.141 | -28.95 | 9.37 | |
| 5 | maize_1 | 2.1640 | Sam | 41.293 | 1.763 | -11.55 | 3.76 | |
| 6 | Maize_2 | 2.1040 | Sam | 41.167 | 1.708 | -11.62 | 3.36 | |
| 7 | Maize_3 | 2.0100 | Sam | 41.331 | 1.680 | -11.73 | 3.95 | |
| 8 | Caffeine_1 | 0.1280 | Sam | 50.173 | 27.617 | -35.23 | -33.59 | |
| 9 | Caffeine_2 | 0.1300 | Sam | 49.997 | 27.691 | -35.43 | -33.61 | |
| 10 | Caffeine_3 | 0.1340 | Sam | 50.546 | 26.654 | -35.30 | -33.56 | |
| 11 | Caffeine_4 | 0.1300 | Sam | 50.981 | 27.257 | -35.37 | -33.57 | |
| 12 | Caffeine_5 | 0.1420 | Sam | 50.400 | 26.382 | -35.49 | -33.36 | |
| 14 | LCP3_6 | 30.0760 | Sam | 1.605 | 0.131 | -29.05 | 7.71 | |
| 15 | LCP3_7 | 30.2840 | Sam | 1.599 | 0.127 | -29.04 | 8.33 | |
| 16 | Caffeine_6 | 0.1420 | Sam | 51.418 | 28.143 | -35.43 | -33.98 | |
| 17 | Caffeine_7 | 0.1400 | Sam | 48.862 | 26.257 | -35.37 | -33.46 | |
| 18 | Caffeine_8 | 0.1380 | Sam | 50.871 | 27.502 | -35.43 | -32.98 | |
| 19 | Maize_4 | 2.0260 | Sam | 41.259 | 1.655 | -11.68 | 2.92 | |
| 20 | Maize_5 | 2.0840 | Sam | 41.606 | 1.823 | -11.55 | 3.17 | |
| 21 | Maize_6 | 1.9760 | Sam | 41.462 | 1.738 | -11.60 | 5.53 | |
| 22 | Caffeine_9 | 0.1500 | Sam | 51.998 | 29.122 | -35.37 | -33.60 | |
| 23 | lcp3_8 | 29.7040 | Sam | 1.591 | 0.137 | -28.41 | 8.32 | |
| 24 | Caffeine_10 | 0.1460 | Sam | 48.742 | 27.451 | -35.31 | -33.68 | |
| 25 | lcp3_9 | 30.4960 | Sam | 1.575 | 0.114 | -28.38 | 8.73 | |
| 26 | Caffeine_11 | 0.1440 | Sam | 51.104 | 27.371 | -35.18 | -33.12 | |
| 27 | lcp3_10 | 30.1600 | Sam | 1.611 | 0.134 | -28.18 | 8.05 | |
| 28 | lcp3_11 | 30.9900 | Sam | 1.590 | 0.123 | -28.02 | 8.35 | |
| 29 | lcp3_12 | 30.0920 | Elem | 1.590 | 0.120 | | | R |

IsoPrime-EA User Manual

End Of Batch Data Processing Results

The report is divided into 3 main areas.

- **Main headings**

Data file and autorun file names are the same as the autorun batch file name specified when setting up the batch.

Blank subtraction Enabled or disabled

Background subtraction Enabled or disabled. Should have no affect on the EA as background is constant

Reference gas Enabled or disabled according to whether isotopic references are included and declared as such.

Reference gas delta Reference gas values as declared in the method file.

Current time/date Always updated whenever a post data reanalysis is carried out

Elemental calculations using TCD areas Quantitative measurements using the TCD. Selected by ticking the "Use TCD Data" option in Batch data processing setup.

Average sensitivity for C or N (Vs / mg) The integrated TCD signal area produced per mg of C/N.

Elemental calculations using mass spec areas Quantitative measurements using the mass spec beam area. This is the normal default setting.

Average sensitivity for C or N (As / mg) The integrated mass spec beam area produced per mg of C/N.

- **Sample detail headings**

The number and type of headings associated with the data are obviously related to the sample analysis involved. Nitrogen analysis, carbon analysis, carbon and nitrogen analysis headings will vary slightly from each other. Nitrogen analysis will provide an atom% heading where carbon analysis does not.

- **Sample Data Output**

The number of columns displaying data depends on the analysis options chosen. All of the various options will include No, Name, Weight, Ref type information. If elemental references are included and named as such in the ref type column then quantitative details will also be shown. N and C isotope data will be shown if peakjumping is used and data on both N₂ and CO₂ has been acquired by the mass spectrometer. An elemental reference will not display the isotopic value. Instead this field will be left blank and the letter 'R' printed at the side. Blanks, labelled as blanks, will have 0.00 printed with the letter B at the side. Nitrogen data will have an atom% output. At the moment Atom% is not available on CO₂. Delta O-18 results for CO₂ can be output in the batch summary report by enabling the general parameter option 'Show O18 results'. Select analysis, parameter file edit, general parameters, edit, show O18 results -TRUE.

Spreadsheet transfer of summary report

The data summary report will be saved in the C:\isotech\EAxx\Autorun directory as a spreadsheet compatible .CSV file. If a spreadsheet is loaded onto the system then the batch .CSV file can be accessed from it. If the spreadsheet is remote from the system then copy the batch.CSV file to a floppy disc for transfer.

Batch DP post data analysis

The batch DP facility features extensive post autorun data processing. Options range from the relatively simple ability to rename samples, specify sample types, correcting errors in sample weights, to the relatively complex, such as correcting some timing errors associated with the method file, examining individual sample integration limits, and drift correction of results.

Note:

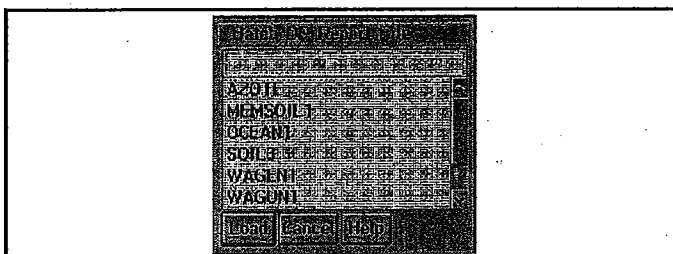
This section will be written in terms of how and as much as possible why, the operator accesses the various options associated with batch DP.

Batch DP options

The following example illustrates quantitative and isotopic measurements on samples containing small concentrations of Carbon.

Select **Batch DP**

All of the previously run batch report files will be displayed. Select the one of interest i.e. MEMSOIL1.



LOAD MEMSOIL1

The batch data processing set up window appears with the following options.

- **Blank subtraction enabled**

If blanks are incorporated into the batch and the sample type is declared as a blank then selecting this option will apply the blank correction to the result summary data.

- **Background subtraction enabled**

This option should have no effect on the data, as in a typical EA run the background is constant.

- **Reference gas enabled**

Tick this option if the delta values of the samples are to be calculated relative to the reference gas. The box should not be ticked if isotopic references are included in the autorun and their sample type is declared as such. If isotopic references are included and declared but the box is ticked then results will be calculated with respect to the reference gas.

IsoPrime-EA User Manual

- Use TCD data

Tick this option if quantitative elemental analysis is to be calculated by integration of the elemental analyser thermal conductivity detector. Otherwise the quantitative analysis will use the integrated mass spectrometer beam areas.

- Batch data_ logger

If this option is ticked then the data logger text file will be overwritten every time the summary report is re-analysed.

- Print report

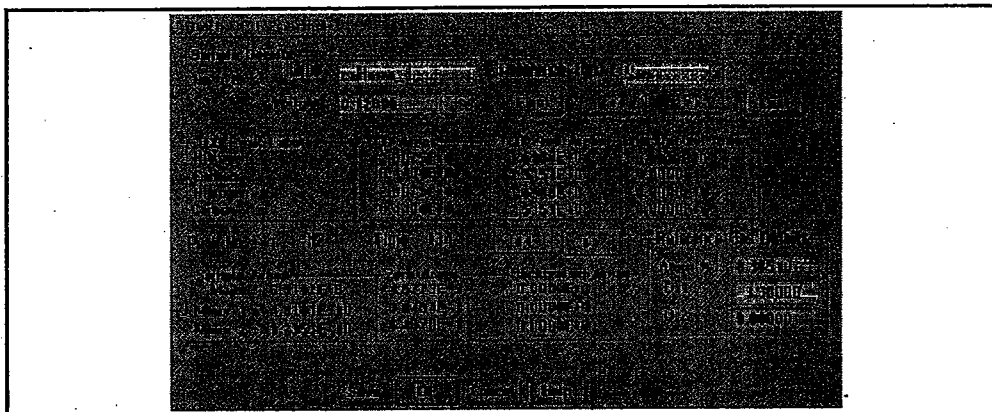
Selecting this option will print the summary report.

- RE-CALC

The results summary can be recalculated, incorporating any global changes such as enabling blank subtraction or after changing some aspect of an individual sample.

- EDIT DETAILS

This option accesses the batch DP re-analysis file. The name of the sample, its type, weight, the reference gas details can all be edited and saved from this window. Also included in this window is direct access to the DP window previously described under manual DP. See section on Introduction to manual data processing.



The top section of the window includes user re-definable sample name, weight, and reference type. Below the reference type box are 4 buttons which enable the samples to be selected. Pushing 'FIRST' selects the first sample in the batch, 'LAST' the last sample. Samples in between are accessed using the right and left pointing arrowed buttons.

The centre section shows the acquired TCD sample areas with their associated background areas. The units of TCD areas are volt seconds. The TCD labels, N and C, are duplicated when peakjumping due to the way the software treats the chromatogram as a nitrogen peak with 2 TCD peaks and a carbon peak also with 2 TCD peaks.

The lower section of the window begins with a peak identification and the time it appears on the chromatogram. To access the second peak when peakjumping is involved, use the arrowed buttons to the right of the time. The sample areas are in units of ampseconds. To the right is the reference gas details box. A change made in the delta value of the first sample will apply to all of the samples.

IsoPrime-EA User Manual

When a change is made to any of these sample attributes they must be saved immediately by pressing the **SAVE** button before moving to the next sample.

If a change is made, for example, changing to a different elemental reference, pressing **cancel**, then **recalc**, will redo the batch summary report incorporating the change.

The DP button

The section Checking the method file parameters introduced the feature **Manual DP** which enables the timings and settings associated with the method file to be checked when running single samples. **Batch DP** is an extension of this feature that allows samples, incorporated into an autorun batch, to be reprocessed, by accessing the DP feature from within the **ISOCHROM EA** software. There are a number of possible reasons why problems may occur in the acquisition of data such as timings, associated with the method file most of which can be investigated using the DP button on the batch DP re-analysis page.

● Example 1

The main summary shows that data has not been acquired from the blanks, making blank subtraction impossible. Move to the sample in question. Select the DP button.

Select **File and Initialise parameters**

Select **Lines** and deselect 3/1, 2/1, minor2, minor1

i.e. Look at TCD and mass spec major only

Select **View** and choose zero

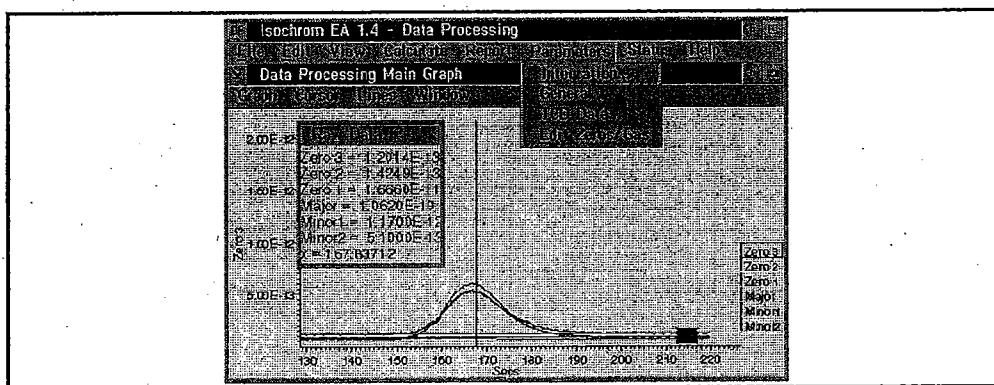
Select **lines** and deselect zero3, zero2

i.e. Only look at the major background zero

Select **Cursor** and choose the **Zoom in** option

Zoom in on both the TCD and mass spec around the time of the expected peak.

Select **Cursor** and choose **Raw data**



Note the time of the peaks, the height of the peaks, the height of the background zero.

Select **Parameters** choose **integration** select **Sample**

Check **min peak to baseline** and **min peak to zero** are set such that the small blank peak does actually meet the conditions. If not then re-edit the values.

IsoPrime-EA User Manual

Try changing the course search step to a different value, say from the default value of 10 to 15 or 20.

Select **Parameters** choose **TCD Data**. Check the timings and also that the peak threshold is set correctly.

Select **Parameters** choose **EDIT Zeros/gas**. Change the background zero positions if necessary, i.e. Edit zero details, left, right.

Whenever a change has been made, return to the chromatogram, select **calculate** and **re-analyse**. A single run report will be generated which will show if the changes made any difference. If the peak has now been acquired then double click on the **DP** button, top left on the data processing window. This action will exit the **DP** and return to **Batch DP** re-analysis, updating the data contained in the file for the blank.

Immediately select the **Save** option.

Cancel

Recalc

The system will provide an updated summary report incorporating the recovered data from the blank file.

Example 2

Samples have been run in triplicate and occasionally there is the odd set where one isotopic delta result is very different to the other two. Unlikely to be a problem with the sample itself or the elemental analyser so it would be interesting to check where the software set the integration limits for that sample.

Move to the sample in question and select **DP**.

Select **File** and **Initialise parameters**

Select **Lines** and **deselect 3/1, 2/1, minor2, minor1**
i.e. Look at TCD and mass spec major only

Select **View** and choose **Sample Printed** beside the peak will be the original calculated delta value.

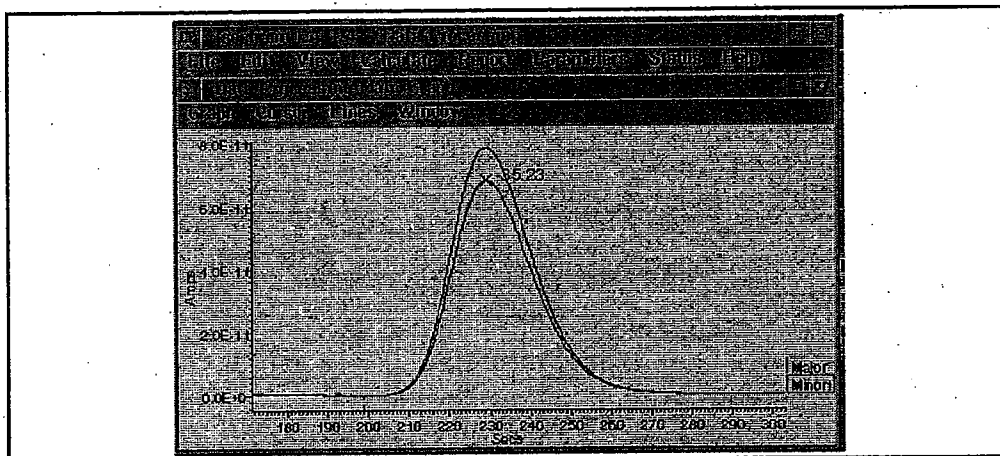
Zoom in to the base of the peak. The integration limits for this sample as defined by the software will show as vertical dotted lines either side of the peak. By inspection it can be determined whether the positions defining the window are sensible or not. In 99.9% of cases they will be. However if it is found necessary to redefine this window.

Select **Cursor** and choose the **drag** option.

The cursor will change to a cross hair. Position over the left or right vertical limit, hold the left mouse button down and drag the limit to a new position.

Select **Calculate** and choose **Re-analysis**. The system will recalculate the delta using the new integration window.

Exit and save as in example 1



Example 3

When comparing standard reference materials placed at the beginning and end of an autorun and the delta values have shifted slightly, it is possible to disable the reference gas calculations and use instead the standard materials to recalculate the run.

Go to the Batch DP Re-analysis page and move to the reference material sample at the beginning of the run. Change its **Reference type** from **Sam** to **Iso**. Select the edit option and define the delta value associated with the reference material. **Close** and **save**.

Move to the reference sample at the end of the run and repeat as above.

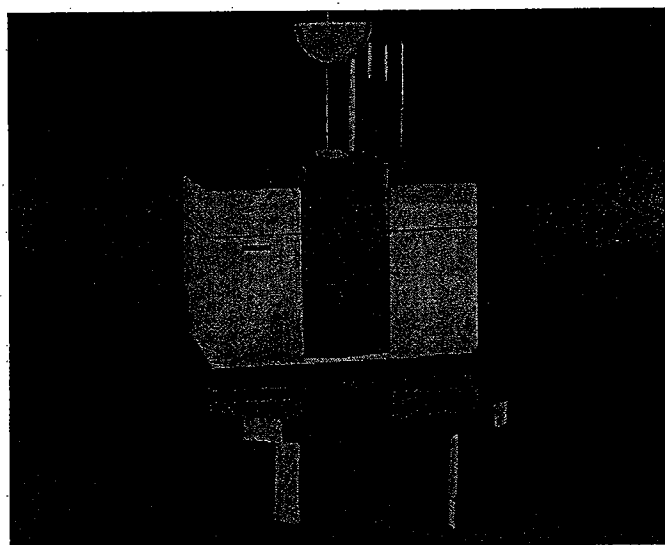
Cancel out of the DP Re-analysis window to the **Batch Data Processing setup** window. Disable the **Reference gas enabled** by removing the tick in its selection box.

Select Re_calc

The data will be re-analysed using reference standards combusted in the elemental analyser.

IsoPrime EA User Manual
Code No 6666588
Issue 1a

Section 7



Advanced Software

IsoPrime-EA User Manual

IsoPrime-EA User Manual

| | |
|---|-----------|
| Introduction | 5 |
| System Files | 5 |
| Editing and Creating Files | 5 |
| Parameter Files | 5 |
| Format | 6 |
| Text Files | 6 |
| CFMIMIC.Txt | 7 |
| Mimic Diagrams (*.TXT)..... | 7 |
| User Menus | 8 |
| Using CSV Format Files..... | 9 |
| Compressing and Archiving Data..... | 10 |
| Programming The IsoPrime-EA | 10 |
| Program Language Syntax | 10 |
| Commands..... | 10 |
| Read Statements | 11 |
| Declarations..... | 11 |
| String Handling Commands..... | 12 |
| Assignment Statements | 12 |
| Program Flow Control | 13 |
| Input/Output | 14 |
| Communication with Communication (Com) Ports..... | 14 |
| Communication Routine Parameters | 15 |
| Miscellaneous | 16 |
| An Example Program..... | 18 |
| Good Practice When Programming | 19 |
| Running a Program | 19 |
| Editing a Program | 19 |
| Testing a Program..... | 19 |

IsoPrime-EA User Manual

Introduction

The purpose of this chapter is to describe the remaining features of the software package. As such it is essentially an Advanced User's guide, which describes briefly aspects of the package which enable users to 'customise' the appearance and operation of their software.

Descriptions are also included of the interface to 'add-in' features such as the 'ISOLINK' remote modem support package.

System Files

Various files are used by the software to set-up the behaviour and layout of the system. These include files to define the appearance of pull-down menus, mimic diagrams, and parameter files. Further files are used to define the programs used to run samples in an Autorun, and perhaps most interestingly for the advanced user, files can be written which allow full programming control of instrument and sample preparation system operation.

Editing and Creating Files

The simplest way to edit system files is with the OS/2 System Editor. If this is not on the OS/2 Desktop, it can be found by opening the 'Productivity' Folder in the 'OS/2 System' folder. Alternatively, open an OS/2 Window from the Command Prompts, and type 'E' at the C: prompt.

Note: The facilities available in the OS/2 System Editor are fully described in the OS/2 manuals, which should be referred to in this section.

When saving a file, the user will be prompted to define a file type. All files should be identified as 'Plain Text', which are simple ASCII Text Files. Use the Save-As command to save the file in the appropriate directory and always use the correct filename extension:

| | | |
|-------|---|------------------------------|
| *.SEQ | - | sequence files |
| *.PRM | - | parameter files |
| *.TXT | - | menu and Mimic diagram files |

It is also possible to create system files using a more sophisticated package, such as Microsoft Word. However it is essential to save such files as **Text only**, as the software will not expect to encounter any hidden formatting characters.

Parameter Files

The values of any of the system parameters can be changed from the Analysis menu, using the Parameter File Edit commands. The actual files however contain much more information. Parameter Files are found in the directory C:\ISOTECH\EA\PARAMS. Their filenames have the form *.PRM.

Format

An example of the format is shown below:

```
1 'Manifold Parameters'
'20 Sample - B Bank Fitted' = FALSE BooleanT 1
'Use Crackers' = FALSE BooleanT 1
'Pumpout Time' = 60 IntegerT 1
'Sample Transfer Time' = 30 IntegerT 1
'Sample Release Time' = 15 IntegerT 1
```

The first line is the text which will appear in the Parameter Files list box on selection of the Parameter File Edit command in the user interface. It is preceded by a number which indicates the minimum User Level needed to access the file from software (see user levels in the User Interface Section of this manual).

Note: The text must be included in quotation marks ('').

Each subsequent line details one parameter, and has the general form:

'Text' = Value, Type, User Level

Text

This must be included in quotation marks, and is the text which will appear in the Parameter File Edit dialogue box. This may be written in a foreign language if required. This text is also used by the IMPORT and EXPORT commands (see later).

Value

This is the value initially assigned to the parameter. It must be in the appropriate format and range for the type of variable used.

Type

This declares the parameter type. Valid selections are:

| | | |
|-----------|---|------------------------|
| BooleanT | - | logical, TRUE or FALSE |
| IntegerT | - | integer numbers |
| RealT | - | real numbers |
| CardinalT | - | Cardinal numbers |
| StringT | - | string of characters |

User Level

A number from 1 to 3 declaring which levels of user have access to changing the parameters.

Text Files

The appearance of Mimic Diagrams on your screen is controlled by a set of files found in the

C:\ISOTECH\EA\SETUP\ directory

All these files have names of the form *.TXT.

CFMIMIC.Txt

This file contains a list of the mimic diagram file to be used on the system.

Mimic Diagrams (*.TXT)

These contain details of the icons for valves, bellows, pipework, thermocouples, pressure gauges, etc., which appear on screen.

The Isochrom EA example below demonstrates the principles involved.

| Isochrom Elemental Analyser | |
|------------------------------------|----------------------|
| Object GC 200 72 | ELEMENTAL ANALYSER |
| Object MassSpec 52 47 | MASS SPEC |
| Mnemonic J5 Valve 190 125 Vert | START VALVE |
| Mnemonic J4 Valve 220 125 Vert | STOP VALVE |
| Mnemonic XD Interface 235 135 Horz | STOP WORD |
| Mnemonic XE Furnace 205 135 Horz | START WORD |
| Mnemonic RG Valve 125 37 Horz | RG (CO2 VALVE) |
| Mnemonic RN Valve 125 13 Horz | RN (N2 VALVE) |
| Mnemonic VY Valve 125 105 Horz | VY (DILUTER VALVE) |
| Mnemonic HS Valve 150 160 Vert | SAMPLE WINDOW |
| Mnemonic IM Valve 100 160 Vert | PENNING GAUGE ENABLE |
| pipe 00 100 82 190 85 | EA HORIZONTAL |
| pipe 00 100 40 160 43 | RG HORIZONTAL |
| pipe 00 100 15 160 18 | RN HORIZONTAL |
| pipe 00 100 70 103 110 | SAMPLE VERTICAL |
| pipe 00 100 15 103 55 | REF VERTICAL |
| pipe 00 70 67 103 70 | MS SAMPLE PIPE |
| pipe 00 70 55 103 58 | MS REF PIPE |
| pipe 00 100 107 160 110 | VY HORIZONTAL |

The first line is text, which appears on the Title Bar of the mimic diagram window. This is followed by a line which describes each icon to be drawn in the window, with each icon corresponding to a mnemonic (which must be a valid mnemonic for your system). Three types are available, illustrated below in example lines.

Mnemonic RG Valve 125 37 Vert

Tells the system to draw a valve icon at co-ordinates x=125, y=37 with respect to the 0, 0 corner of the mimic diagram window. This will correspond to Mnemonic RG, and will be labelled as such on the window.

Note: Valves can be shown in either vertical 'Vert', or horizontal 'Horz' orientation, to better illustrate the connectivity of the system.

PIPE n x1 y1 x2 y2

Icons drawn in the mimic diagram window can be shown connected by lines of varying thickness. The command in the *.TXT file takes the form:

The number n is not currently used. The co-ordinate pairs x1 y1 and x2 y2 define a rectangular area of pixels with respect to the 0, 0 position of the window. This will be filled in grey to form a pipe or connection on the finished screen display.

User Menus

It is possible to add customised commands and procedures (written as *.SEQ files) to certain of the pull down menus.

The files C:\ISOTECH\EA\SETUP\USERMENU.TXT contains a list of editable commands on the Inlet, Mass Spec, and Prep menus. An example is shown below. The file contains one line for each added menu command.

Each line has the following format:

Text : number : *.SEQ : optional parameters :

Text is the text which will appear on the pull down menu. If a character is preceded by a tilde (~), then that character will appear underlined, and can be used for keyboard operation via the 'alt' key. Note that the text may be written in a foreign language if required.

Number is a key to the menu on which the command will appear. Valid selections are

- 2 - Inlet menu
- 7 - Mass Spec menu

***.SEQ** is the name of a valid sequence file in the C:\ISOTECH\EA\SEQUENCE directory. This sequence will be executed when the menu command is selected.

Optional Parameters are added if the named *.SEQ file expects to receive parameters, they should be placed here, separated by either spaces, or commas.

Note: If no parameters are passed, the line has the form.

Text ; number ; *.SEQ;;

Using CSV Format Files

The **Datalogger** facility in the software suite stores raw results information in files where each data item is separated by a comma. This gives rise to the term CSV (Comma Separated Variables). Such files can be imported into third party suites for further processing.

The details of how to read in such files will vary from one spreadsheet package to another. The example below describes how to import a file into Microsoft Excel 5.0 running under Windows.

From the **File** menu, choose the **open** command.

Set the directory to **C:\ISOTECH\EA\DATA**

From the '**List Files of Type**' box, choose the **Text Files** options.

Click on the file name, then **OK**.

The file will now be imported into Excel.

Note: Because the software is multitasking, the spread sheet programs can be used while the IsoPrime is carrying out analytical work, maximising the productivity.

Compressing and Archiving Data

If the Datalogger system is being used to store the raw data, it is good practice to copy the files onto media which can be stored in a safe place.

Files may be copied from the C:\ISOTECH\EA\DATA directory onto the a:\ drive using either the Drives program from the OS/2 system folder, or perhaps more easily using the copy command from an OS/2 Window e.g.:

COPY C:\ISOTECH\EA\DATA*.TXT A:

would copy all *.TXT files into the current directory on the disk in drive A.

If the data is required compressed it is recommended that a more sophisticated compression package such as 'PKZIP' or 'LHA' is used.

Programming The IsoPrime-EA

One of the unique features of the IsoPrime-EA suite is the incorporation of a powerful programming facility. This enables the user to completely program the operation of sample preparation systems, control valves, stepper motors, read all analogue outputs from the instrument (including ion beams), and to set all digital registers available via the mnemonics (e.g. retuning the ion source from a program). The language includes all arithmetic operations, real, integer and logical variable types, conditional statements, IF's GOTO commands, labels, subroutine calls etc.

In short the objective is to provide all the flexibility of an interpreted language like BASIC whilst retaining all the power and facilities of the operating system and the compiled source code.

Program Language Syntax

Commands

Commands available are :

OPEN mnemonic

Opens the valve identified by mnemonic

CLOSE mnemonic

Closes the valve identified by mnemonic

START mnemonic

Turns on the switch identified by mnemonic

STOP mnemonic

Turns off the switch identified by mnemonic

ANOUT mnemonic,value

Sets the specified analog output to the desired value

STEPOUT mnemonic,value

Tells the stepper motor identified by mnemonic to move to position identified by value

Read Statements

Read Statements available are :

READBEAM mnemonic, variable

- Reads the specified ion beam, the mnemonics are:
 - J0 Major
 - J1 Minor 1
 - J2 Minor 2
 - J3 Minor 3

READMOTOR mnemonic, variable

- Reads the position of the stepper motor identified by mnemonic and stores the result in variable.

READVALVE mnemonic, variable

- Reads the state of the valve and stores the result in the variable (Boolean)

DIGIN mnemonic, variable

- Reads the digital signal identified by mnemonic and stores the result in variable

ANIN mnemonic, variable

- Reads the analog signal identified by mnemonic and stores the result in variable (which must be real).

- For example:

- ANIN T1,FINGERTEMP

- Reads thermocouple 1 (the cold finger) and stores the result in FINGERTEMP

Declarations

Declarations available are:

INTEGER list of variables

- Declares a list of variables as of integer type: an integer can be in the range -2,147,483,648 to +2,147,483,647

REAL list of variables

- Declares a list of variables as of real type. A real can be in the range +/- 2.3E-308 to +/-1.7E308 with 15 digits of precision.

BOOLEAN list of variables

- Declares a list of variables as of Boolean type (i.e. Logical) type. A BOOLEAN variable may adopt the value TRUE or FALSE

FLAG list of variables

- Declares the variables as GLOBAL booleans. This means that they will be valid in all programs which are running concurrently.

STRING list of variables

- Declares a list of variables as of string (i.e. text/character) type. STRING variables may be up to 80 characters long.

String Handling Commands

APPEND StringVariable StringVariable/String

- Appends the second parameter to the first string.

PREPEND StringVariable StringVariable/String

- Prepends the second parameter to the first string.

COMPARE BooleanResultVariable StringVariable StringVariable/String

- Compares the first string to the second and if they are the same then returns true.

SLICE StringVariable StartPosition Length StringVariable/String

- Copies the portion of the last string from the 'start position' for the next 'length' characters into the first string.

VALSTR String ASCIIValue

- Converts the ASCII value into a character and returns it as a string.
- **Note:** ASCII codes can be used in String commands APPEND, PREPEND and ASSIGN by preceding the number with the @ symbol (e.g. ASSIGN Cr @10 would put the ASCII text for the character 10 into the string Cr).

STRVAL ASCIIValue StringVariable/String

- Returns the ASCII value of the first character in the string.

Assignment Statements

Assignment Statements available are:

ASSIGN variable,value

- Sets variable to value

ADD variable, value1, value2

- Adds value2 to value1 and stores the result in variable

SUBTRACT variable,value1, value2

- Subtracts value2 from value1 and stores the result in variable

MULTIPLY variable,value1, value2

- Multiplies value1 by value2 and stores the result in variable

DIVIDE variable, value1, value2

- Divides value1 by value2 and stores the result in variable

OR Variable Boolean1 Boolean2

- Performs a logical OR on the 2 booleans and stores the result in the variable

NOT Variable Boolean1 Boolean2

- Performs a logical NOT on the booleans and stores the result in the variable

AND Variable Boolean1 Boolean2

- Performs a logical AND on the booleans and stores the result in the variable

Program Flow Control

Program Flow Controls available are:

IF value1, conditional, value2, label

- Compares value2 to value1; if the conditional operator ($=$, $>$, $<$) is satisfied, the statement is TRUE, and execution will proceed at the line following "label". Otherwise execution will proceed at the next line.

:label-name

- A valid label name is an alphanumeric string preceded by a colon. Label-names are referred to by GOTO and IF statements.

GOTO label

- Execution proceeds from the line after the label referred to.

EXIT ReturnVal

- Ends the program, returning the real variable ReturnVal to the calling program. The last line of the program must be an EXIT.

CALL return variable SEQUENCE FILENAME <parameters>

- Starts execution of the program specified by SEQUENCE FILENAME. Any required parameters must be passed. The execution of the calling program is suspended until the called program executes an EXIT statement. The Return value of the EXIT statement is stored by the calling program in return variable.

SPAWN SEQUENCE FILENAME <parameters>

- Starts execution of the program specified by SEQUENCE FILENAME. Any required parameters must be passed. The execution of the Spawning program continues, so that the two programs run in parallel. No return variables are possible.

WAIT value

- Waits for value seconds before proceeding with the program. Value may take the form of a suitable variable name.

Input/Output

Input/Output available are:

MESSAGE colour number String

- Sends a text message to the message window. The message is shown in the specified colour. String contains the text to be output. The contents of a variable can be output by specifying the variable name preceded by a \$ (e.g. MESSAGE -1 Value is \$Val would output the contents of a variable Val).

- Note: Upto five variables can be used in one message.

- The main colour numbers are:

| | | | | | |
|----|------------|---|-----------|----|------------|
| -2 | White | 4 | Green | 10 | Dark red |
| -1 | Black | 5 | Cyan | 11 | Dark Pink |
| 0 | Background | 6 | Yellow | 12 | Dark Green |
| 1 | Blue | 7 | Neutral | 13 | Dark Cyan |
| 2 | Red | 8 | Dark Grey | 14 | Brown |
| 3 | Pink | 9 | Dark blue | 15 | Pale Grey |

- By convention, red should be reserved for error or warning messages.

QUESTION Answer_Variable String

- Opens a question dialogue box on the screen. String contains a prompt to the user which requires a yes/no response. If the response is Yes, the Boolean answer_variable is set to TRUE, if no it is set to FALSE. This enables the user to control the flow of execution of the program.

Communication with Communication (Com) Ports

SETUP Comport BaudRate Parity DataBits Stopbits
SoftwareHandshaking HardwareHandshaking

- Opens the communication port with the specified settings

PUTPORT ErrorVariable PortMessage

- Writes one character to the communication port:

READPORT ErrorVariable Port Message

- Reads a string from the communication port. This will read until the terminator string is returned.

GETPORT ErrorVariable Port Message

- Writes one character to the communication port then read one character immediately.

WRITEPORT ErrorVariable Port Message

- Writes a string from the communication port.

READPORTFILEIN ErrorVariable Port FileName

- Reads text from the communication port and writes it out to the file C:\ISOTECH\DI\FileName.PRT. It will keep reading until the terminator is received.

WRITEPORTFILEOUT ErrorVariable Port FileName

- Reads the text file C:\ISOTECH\DI\SETUP\FileName.PRT and writes it to the communication port.

Errors

- The values returned as errors from the communication port are as follows:
 - 0 - OK
 - 1 - Unknown Error
 - 2 - Cannot read from or write to the port

Communication Routine Parameters

Maxresp

- Is an INTEGER with a default of 50 characters to read (Note: 0 will give as many characters as needed).

Timeout

- Is an INTEGER with a default of 10000 milli-seconds to wait for a response.

WaitTime

- Is an INTEGER with a default of 100 milli-seconds to wait before re-trying the communication port.

Retry

- Is an INTEGER with a default of 1 times to re-try writing to the communication port.

RdTerm

- Is an STRING with a default value of CrLf. The communication port will be read until the read terminator is received.

WrTerm

- Is an STRING with a default value of CrLf. The communication port will be written to until the write terminator is sent.

If any other settings are required declare the variable of the correct type and assign the required value.

Note: This must be done in each sequence using the communication port commands. If any variables are undeclared the default value is used.

Miscellaneous

Miscellaneous commands available are:

JUMP value HTJump PeakCentre

- Jumps to the mass specified by value, using HT if HTJump is set TRUE or by Magnet if HTJump set FALSE. If PeakCentre is set TRUE it will perform a peak centre after the jump.

LOAD Tfname 255

- Retunes the ion source by loading the specified tuning file. The 255 is a bit set value which specifies which of the source parameters to load (e.g. 1 = HT only, 4 = Half Plates only, etc.).

Mnemonics

- Mnemonic 2 letter identifiers for valves switches etc.

string

- Any set of alpha-numeric characters.

Variable Names

- Variable Names consist of a string, started by a letter, of between 3 and 12 characters. The names are case sensitive.
- FINGtemp and FingTEMP are different variables.
- Before use, a variable name must be declared in a REAL, INTEGER, or BOOLEAN declaration statement.

Parameters

- A parameter is identified by a % symbols
- e.g. WAIT %0
- Will use the value passed in to the sequence as the first parameter.

Comments

- A line is treated as a comment if its first character is %

IMPORT variable name, '*.PRM', 'Text'

- Will read the value of the parameter identified by 'Text' from the parameter file *.PRM, and store the result in variable name. This command thus enables sequences to import values from parameter files.
- **Note:** If, and only if, a variable is imported from a parameter file, it does not need to be declared in an BOOLEAN, REAL, INTEGER etc. statement. This is because the parameter file will define the variable type.

IsoPrime-EA User Manual

EXPORT variable name, '*.PRM', 'Text'

- Will write the value of variable name to the file *.PRM, stored on the new value of the parameter identified by 'Text'.
- **Note:** variable must be of the same type as that defined in the parameter file.

PARK mnemonic

- Where mnemonic is a valid stepper motor, this command steps the motor device back to its zero reset position, as controlled by the appropriate expanded position.

CONTROL TEMP Mnemonic Setpoint Duration.

- This will allow the device identified by Mnemonic to be controlled at Setpoint (°C) temperature for the specified duration (minutes).

CONTROL TEMP STATUS Mnemonic Status Variable name

- This will query the status of the temperature device identified by Mnemonic. Status values returned are
 - -998 - no control in progress
 - -999 - device tripped
 - Other value - temperature reading (°C)
- and will be stored in the Integer variable given as status variable name.

ABORT CONTROL TEMP mnemonic

- This will abort a temperature control operation.

IDENTIFY MASS

- This will update the current mass, setting the relevant beam mappings. Mass should be the mass of the species to identify.

BEEP frequency duration

- This will sound the computer speaker

Parameters are:

frequency – an integer value representing the frequency (Hz) of the tone to be sounded.

Duration – an integer value representing the time (ms) that the tone is to be sounded for.

An Example Program

```
% An example program
% This program illustrates some of the features available
% It monitors the pumpout of the reference gas by measuring the major beam
intensity. If it does not fall sufficiently in a given time, the user is asked if they
want to try again
% Declare variables
INTEGER Pumptime,Counter
REAL Majorbeam,Allgone
BOOLEAN Answer

% Initialise variables
ASSIGN Pumptime, 60
ASSIGN Allgone 1.0E-12
ASSIGN Counter,0

% Set valves to pump out reference
OPEN LV
OPEN RP
OPEN RF
OPEN RV
OPEN RM

% Set changeover valve to reference
CALL Dummy SETCHOV.SEQ TRUE

:Pumpout
ASSIGN Counter,0

:Loop
WAIT 5

% Read Majorbeam
ANIN J0, Majorbeam

% Increment the loop counter
ADD Counter,Counter,5
IF Counter < Pumptime Loop

% has the gas been pumped out?
IF Majorbeam < Allgone Finish

% The gas has not all been evacuated
QUESTION Answer Pumpdown too slow -Do you want to continue?
IF Answer = FALSE Quit

% The user wants to retry the pumpout
GOTO Pumpout

:Quit
MESSAGE -1 Program Aborted
GOTO END

:OK
MESSAGE -1 Reference evacuated OK

:END

% Close all valves
SPAWN CLOSEDIV.SEQ
EXIT 0
```

Good Practice When Programming

Make the first line of a program a comment which identifies the author, date of revision and purpose of the program

Declare all variables at the start of the program

Use self-explanatory variable names

Use frequent comments.

Break programs down into subroutines using labels and comments, and use CALL, rather than writing complex programs that are hard to follow and may be too lengthy.

Use integer variables and arithmetic where the precision of real variables is not absolutely needed.

Take back ups of your program on floppy disc.

Running a Program

Choose the PROGRAM, RUN option from the main menu bars

Select the program to run from the dialogue box which appears: remember to enter any necessary parameters using the parameter entry field.

Press OK or Return to start the program.

Editing a Program

A program can be entered on the IsoPrime data system by using the OS/2 System editor, which is a powerful text processor. It has extensive search and replace features and on screen editing facilities, such as cut and paste, which are similar to those provided by any Text processing software. Fonts and text colours are also selectable. A detailed manual for this editor is provided with the system.

Note: Because the software is multitasking, programs can be written while the IsoPrime is carrying out analytical work, maximising the productivity. However be careful not to modify sequences which are currently running.

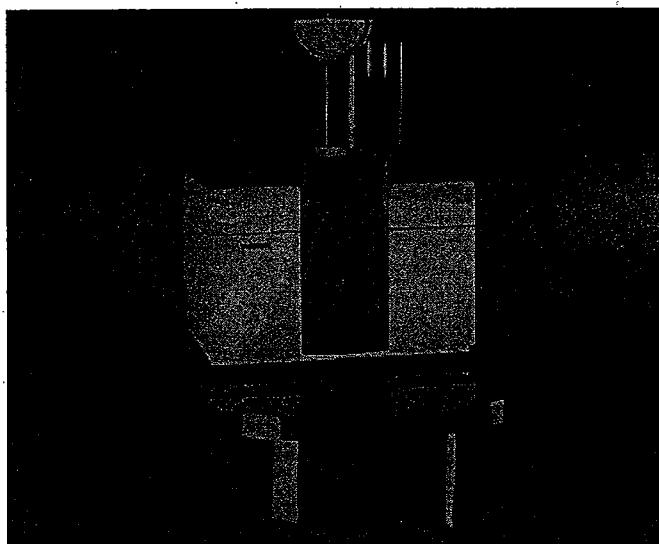
Testing a Program

The best way to test a program is to run it first using the off-line software which is provided with the system. Again, the productivity is maximised, and the programs can be tested without risking damage to the instrument, however sequence commands setting the bellows, motor positions, etc. will obviously not drive the specified devices while running off-line.

IsoPrime-EA User Manual

IsoPrime EA User Manual
Code No 6666588
Issue 1a

Section 8



Maintenance

IsoPrime-EA User Manual

IsoPrime-EA User Manual

| | |
|---|-----------|
| Introduction | 7 |
| Toolkit..... | 7 |
| Useful Tools..... | 7 |
| Emergency fault conditions | 8 |
| Emergency Instrument Shutdown | 8 |
| Electrical power failure..... | 8 |
| Other supplies failure..... | 9 |
| Compressed Air | 9 |
| Supply gases | 9 |
| Helium..... | 9 |
| Oxygen | 9 |
| Reference Gas | 9 |
| Routine Maintenance for the Mass spectrometer..... | 10 |
| Planned maintenance | 10 |
| Change the Rotary Pump Oil | 10 |
| Change the Molecular Sieve in the Foreline Traps | 10 |
| Replacement of molecular sieve without venting the IsoPrime | 10 |
| Change Oil Mist Filter Elements | 11 |
| Compressor (if supplied) Maintenance..... | 11 |
| Compressed Air Water Trap Drainage | 11 |
| Mass spectrometer Repairs..... | 12 |
| Overview..... | 12 |
| Venting the Mass Spectrometer..... | 12 |
| Removing the Ion source | 13 |
| Source flange removal | 13 |
| Ion source removal | 14 |
| Replacing the Ion Source | 15 |
| Fitting the ion source to the source flange | 15 |
| Replacing the source flange..... | 17 |
| Changing the Ion Source Filament | 18 |
| Filament removal..... | 18 |
| Filament positioning | 18 |
| Filament assembly | 19 |

IsoPrime-EA User Manual

| | |
|---|----|
| Disassembly of the Ion Source..... | 20 |
| Magnet, filament and trap removal | 20 |
| Stack disassembly | 20 |
| Disassembly of the ion box | 24 |
| Dismantling the trap assembly | 24 |
| Dismantling the magnet assembly | 25 |
| Assembly of the Ion Source | 25 |
| Folding photo-etched components | 25 |
| Trap..... | 25 |
| Source wrap-around | 26 |
| Filament shield | 26 |
| Reassembling the ion box assembly | 27 |
| Reassembling the trap assembly..... | 28 |
| Reassembling the magnet assembly | 28 |
| Reassembling the stack | 28 |
| Penning Gauge Maintenance | 32 |
| Cleaning Procedures | 32 |
| Rough Cleaning..... | 32 |
| Pre-cleaning | 32 |
| Final Cleaning..... | 32 |
| Penning gauge cleaning..... | 33 |
| Rotary Pump Repair..... | 33 |
| Turbomolecular Pump and Vent Valve Repair..... | 33 |
| Air Compressor (if supplied) Repair | 33 |
| Electronics Repair..... | 33 |
| Removal of the system controller | 33 |
| Refitting the system controller | 34 |
| Removal of the bakeout power supply | 35 |
| Replacement of the bakeout power supply | 36 |
| Removal of the electromagnet power supply | 36 |
| Replacement of the electromagnet power supply | 37 |
| Removal of the head amplifier | 37 |
| Replacement of the head amplifier..... | 38 |
| IsoPrime user replaceable fuse information..... | 38 |
| Mains inlet to IsoPrime | 38 |
| System controller | 39 |
| Bakeout supply..... | 39 |
| Electromagnet supply..... | 40 |
| Computer, Monitor and Printer Repair | 40 |

| | |
|---|-----------|
| Routine Maintenance for the EA | 40 |
| Cutting vitreous silica capillaries | 40 |
| Replacing the reference capillary | 41 |
| Rebuilding the reference gas injector | 42 |
| The reference gas injection assembly | 42 |
| The MOVPT valve | 43 |
| The MCVT-1 splitter valve | 44 |
| Changing Gas Bottles | 45 |
| Helium | 45 |
| Reference gas | 45 |

IsoPrime-EA User Manual

Introduction

This section of the manual will give details of the routine maintenance and fault finding of the IsoPrime-EA. It is important that routine maintenance is carried out on a regular basis, as this will improve the reliability of the instrument and in turn the quality of the results when running real samples. There are two things to bear in mind before attempting any maintenance or fault finding:

- Read the manufacturers guides supplied for the details of routine maintenance for the components of the system e.g. Rotary pumps, Turbomolecular pumps, Air Compressors, Elemental analyser, etc.
- Fault finding and system repairs should be carried out by a suitably qualified technician. Please contact the Customer Service Department at Micromass or the local Micromass representative with any queries.

Toolkit

The IsoPrime is supplied with a toolkit containing the following components.

Pliers, snipe nose.

Eye magnifier.

Adjustable wrench, 6".

Spanner, open ended, 3/4".

Spanner, open ended, 1/2-9/16".

Spanner, open ended, 3/8-7/16".

Spanner, open ended, 1/4-5/16".

Jewellers screwdriver 0.4mm.

Screwdriver, dumpy, flat 5mm.

Screwdriver, flat 3mm.

Hex key 10mm.

Balldriver 3mm.

Balldriver 5mm.

Additional components supplied within the installation kit are:

Ceramic capillary cutter.

Source assembly jig.

Useful Tools

Additional tools that may be of use in performing the routine maintenance tasks described in this section of the manual.

Set of metric Allen keys (hexagon keys)

Pair of tweezers

Lint-free gloves (dental gloves are ideal i.e. non-powdered)

A digital multimeter

Medium size screwdriver (straight head)

Medium size screwdriver (Philips head)

Set of Jeweller's screwdrivers

Emergency fault conditions

Emergency Instrument Shutdown



Warning: Before returning the power to the system ensure the reason for the shutdown is corrected.

In exceptional circumstances (such as fire or exposure to hazardous voltages) it may be necessary to shut down the instrument quickly. If such a situation arises, the instrument should be isolated from the electrical supply, as close to the supply as possible:

- Isolate the supply at the distribution box that connects the instrument and the preparation system to the site supply, if this can be done quickly. This will usually mean operating the isolation lever on the outside of the box.
- Switch off the rotary pump by the toggle switch on its side, and then unplug the mains inlet cables from the rotary pump and the rear of the IsoPrime cubicle.
- Switch off the mains power to the elemental analyser and then unplug its mains inlet cables (see manufacturer's guide for details).
- Disconnect the power cables of any peripherals connected (such as sample preparation systems, etc.).
- When there is no further risk to personnel:
 - Close the rotary pump isolation valve.
 - Turn off the power switches to the PC, Monitor and Printer.
 - Set to zero all the temperature settings on the elemental analyser. (See manufacturers guide)

Electrical power failure



Warning: Repair the Electrical fault before returning the power to the system.

If the power supply to the instrument fails for any reason (e.g. during an electrical storm) the following procedure should be followed:

- Close the rotary pump isolation valve.
- Switch off the rotary pump by the toggle switch on its side.
- Switch off the power to the IsoPrime cubicle, or remove the IEC connector.
- Switch off the mains power to the elemental analyser (see manufacturer's guide for details).
- Turn off the power switches to the PC, Monitor and Printer.
- Set the elemental analyser temperatures to zero. (See manufacturers guide)

When the failure has been rectified:

- Follow the standard power-up procedure for the Pumps/Electronics.
- Follow the standard power-up procedure for the elemental analyser.

Other supplies failure

Compressed Air



Warning: To avoid possible eye injury, wear eye protection when using compressed gas.

If the compressed air fails the valves on the reference gas injector and the GC interface will close and fail to open.

The reason for the compressed air failure should be located and rectified. This could include:

- Check the air compressor (see manufacturers guide).
- Check air lines.
- Check for blockages.

Supply gases

Helium



Caution: Failure of the Helium flow may damage the mass spectrometer, and thermal conductivity detector. The performance of the separation column and combustion tubes may also be significantly affected.

It is essential that the Helium flow not be interrupted whenever the elemental analyser is switched on, or the Nupro isolation valve is open.

If Helium is not flowing constantly, air will enter at the open splits and flow into the mass spectrometer. The source filament may be damaged.

It is strongly advised to carefully monitor the amount of gas remaining in the helium supply bottle, in particular before leaving at night and at weekends!

Oxygen

The continuous availability of oxygen is essential to the operation of the elemental analyser. No damage to the mass spectrometer or elemental analyser will result if the oxygen supply fails but there will be an obvious degradation in analytical performance.

Reference Gas

No damage to the instrument will result if the carbon dioxide or nitrogen supply runs out. However, if this occurs during an acquisition it will not be possible to obtain a Delta calculation with respect to the reference gas.

Carbon dioxide is usually in the liquid phase, when stored in pressurised reservoirs. This makes it difficult to monitor the amount of gas remaining in the bottle. Additionally, as the bottle becomes empty, the gas has a tendency to fractionate, that is, its isotopic composition changes. If this occurs, loss of accuracy will result.

As a rule of thumb, it is wise to change the CO₂ reference gas bottle as soon as the bottle pressure starts to fall. This indicates that only CO₂ in gas phase remains, suggesting that most of the gas has already been used.

Nitrogen bottles should be changed as soon as the recommended outlet pressure of 4 bar can no longer be maintained.

Routine Maintenance for the Mass spectrometer



Caution: Refer to the manufacturers guides supplied, for details of routine maintenance of third-party system components e.g. Rotary pumps, Turbomolecular pumps, Air Compressors, etc.

Planned maintenance

Many of the problems associated with poor instrument performance can be prevented with regular and effective maintenance. It cannot be stressed too strongly that two days service every six months will save time and increase sample throughput in the long term.

The main aim of routine maintenance is to rectify potential problems before they affect the accuracy of the instrument.

The following procedures should be carried out at intervals no greater than **six months**, unless a shorter time period is specified in the manufacturer's manual.

Change the Rotary Pump Oil

A rotary pump is a mechanical pump that relies heavily upon the performance of the oil to provide lubrication and cooling. The condition of the rotary pump oil will also directly affect the pumping ability of the rotary pump. In use the oil slowly breaks down, which in turn reduces pumping efficiency. New oil is a light brown colour, while old oil varies from dark brown to black. Oil which is cream coloured has been pumping a lot of water and the source of this water must be identified before resuming sample analysis. Running with old oil for extended periods of time will contaminate the analyser.

Note: New oil must be ballasted according to the manufacturer's instructions.

Note: Only use the oil specified by the manufacturer. Use of the wrong oil will result in decreased performance and possible damage to the pump.

The oil change procedure is detailed in the manufacturer's manual.

Change the Molecular Sieve in the Foreline Traps

The foreline trap fitted to the rotary pump contains molecular sieve, which traps oil vapour from the rotary pump and prevents it from contaminating the system, so to ignore this procedure will lead to a build up of hydrocarbons in the analyser.

The manufacturer's manual contains details of the procedure for replacement of the molecular sieve.

Follow the manufacturer's suggestions for replacement intervals.

Replacement of molecular sieve without venting the IsoPrime

It is possible to replace the molecular sieve in the foreline trap without venting the instrument by the following procedure.

Note: The whole operation must be performed within 5 minutes or the instrument must be vented.

1. Prepare the new charge of molecular sieve by drying thoroughly in an oven (see manufacturers manual for details).

2. Close the Nupro isolation valve on the IsoPrime.
3. Switch off the source.
4. Close the Speedivalve on the rear of the IsoPrime.
5. Switch off the rotary pump by the rocker switch on the side of the pump.
6. Vent the foreline by opening the purge valve fitted to the top of the foreline trap.
7. Remove the molecular sieve canister and replace the sieve according to the manufacturer's instructions.
8. Refit the canister and re-assemble the foreline trap.
9. Close the purge valve.
10. Switch the rotary pump on and allow it to pump for 1 minute.
11. Open the Speedivalve.
12. Observe the foreline pressure. A value of greater than 1×10^{-2} indicates a leak.
13. Observe the analyser pressure and turbo speed. When both have returned to normal values open the isolation valve.
14. Switch on the source.

Should it prove impossible to replace the foreline trap within 5 minutes, switch off the turbo and allow the instrument to vent. Complete the change of molecular sieve and pump down the instrument as normal.

Change Oil Mist Filter Elements

While this does not necessarily affect the performance of the instrument, changing the Oil Mist filters ensures that the environment the system operates in, is kept as safe as possible for the user.

Details of the procedure for replacement of the oil mist filters are given in the manufacturer's documentation.

Follow the manufacturer's suggestions for replacement intervals.

Compressor (if supplied) Maintenance

The performance of the instrument relies on the smooth running of the valves on the reference gas injector and the Gas Chromatograph. These are pneumatically operated, so the continued performance of the air compressor and airlines is important. The air compressor (if supplied with the instrument) should therefore be maintained in accordance with the manufacturer's guide supplied with it.

Compressed Air Water Trap Drainage

The compressed air water trap (if fitted) should be checked and drained two or three times weekly. Failure to do this may cause damage to occur to the Predyne valves used to operate the valves. Instructions for draining the water trap are included in the manufacturer's guide.

Mass spectrometer Repairs



Caution: Suitably qualified personnel only should perform maintenance operations. Please contact the Customer Service Department at Micromass or your local Micromass representative with any queries.

Overview

This section of the manual will deal with repairs to the system. Care must always be taken when removing items from the system to avoid damage and contamination. It is also advisable for the user to take notes, as the components are disassembled, to complement this manual during reassembly.

The components within the vacuum enclosure are very sensitive to contamination. Precautions must be taken to avoid introducing any contamination during maintenance procedures. It is advised that disposable gloves (unpowdered) be used when handling any of the components from within the vacuum enclosure. A single fingerprint within the vacuum enclosure may be sufficient to affect to operation of the instrument.

Remember: If there are any queries please contact the Customer Service Department at Micromass or the local Micromass representative.

Venting the Mass Spectrometer

Venting the mass spectrometer is basically the reverse of pumping the system down and the following procedure should be carried out:

1. Turn OFF the source using the option on the software.
2. If the ambient atmosphere contains a high level of moisture, it is advisable to connect a source of dry gas (high purity Helium, Nitrogen or dry air) to the outlet of the vent valve.
3. Switch OFF the turbo from the software. This turns off the turbomolecular pump controller.
4. Immediately after switching off the turbomolecular pump, close the backing line isolation valve.
5. The turbomolecular pump will now slow down and after approximately 5 minutes the pump will have reached 50% of full speed. At this point the vent valve will open and the system will come up to atmosphere, as shown by the Pirani gauge in the monitor window.
6. Isolate the mains power to the cubicle or disconnect the IEC connector. The various parts of the system can then be opened as required.

Removing the Ion source



Caution: Wear hand protection (gloves) for this operation to avoid contamination.

Source flange removal



The procedure for removing the source flange is as follows:

Caution: Care should be taken not to bend the feedthrough wires. Failure to follow this precaution may create vacuum leaks on the feedthrough.

1. Follow the procedure above and vent the mass spectrometer.
2. Remove the screws retaining the source connector cover and remove the source connector cover.
3. Disconnect the plugs labelled J1 and J7 from the system controller.
4. Unplug the two half plate leads from the source feedthroughs (labelled 1 and 4). Tuck the two half plate leads into the system controller.
5. Unplug the bakeout connections on pins 6 and 12 (where fitted). Tuck the leads into the system controller.
6. Using the extended 5mm ball-driver in the toolkit, undo the six bolts retaining the source flange to the vacuum housing.
7. If necessary use two of the mounting screws fitted into the jacking screw holes to loosen the flange.
8. Withdraw the source flange a short way and using the pliers from the toolkit, remove the bakeout connector wires on pins 6 and 12 (where fitted).
9. Remove the source flange. Ensure that the quad ring remains in its mounting groove.
10. Place the source flange on a clean lint free tissue on a flat work surface. Protect the sealing faces from abrasion.
11. Cover the open end of the analyser with a clean plastic bag to prevent ingress of contamination during maintenance.

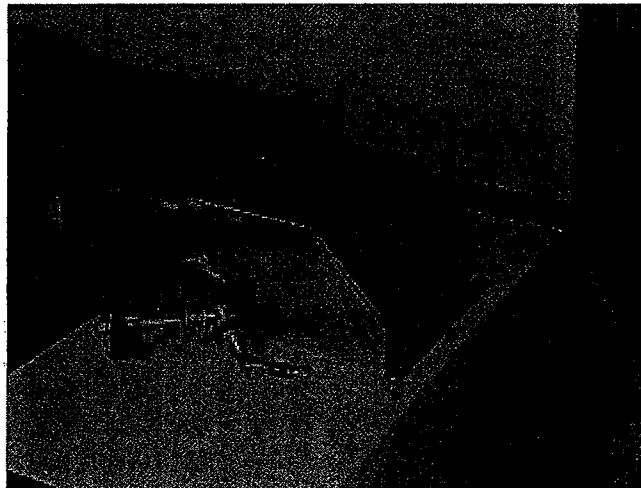


Photo showing source connector cover

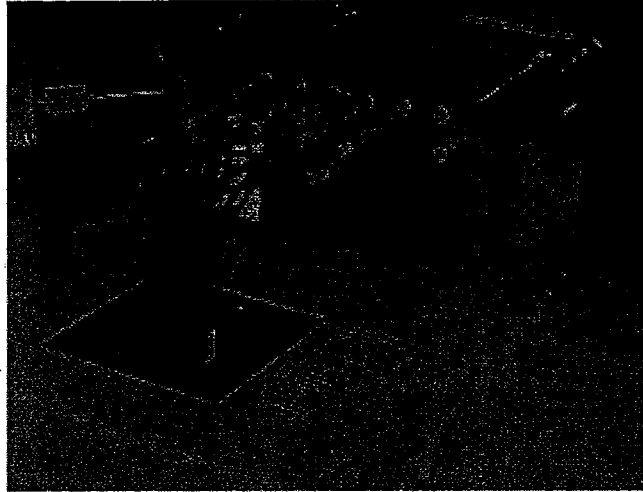


Photo showing source connection leads

Ion source removal

1. Using the pliers from the toolkit pull the gold push fit connectors from the feedthrough pins and push fittings on the source. Remove the filament connections first.
2. Support the ceramic inlet tube whilst retracting the spring-loaded probe into the inlet support. Remove the ceramic inlet tube, spring loaded probe and spring.
3. Undo the two screws holding the source to the source flange.

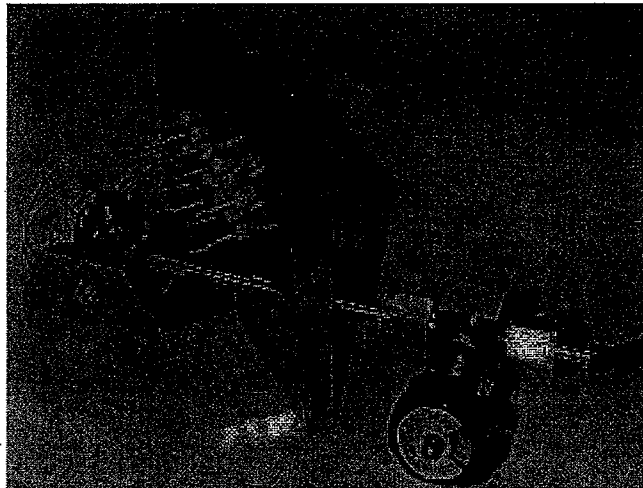


Photo of source mounted on source flange

Replacing the Ion Source



Caution: Wear hand protection (gloves) for this operation to avoid contamination.

Fitting the ion source to the source flange

The procedure for replacing the Ion source on the source flange is as follows:

1. Place the ion source into position on the source flange and secure with the two screws. Ensure that the front face of the source is parallel with the edge of the source flange.
2. Insert the spring into the inlet support and fit the spring-loaded probe. Withdraw the spring-loaded probe and insert the ceramic inlet tube into position in the side of the ion box. Ensuring a good seat in the ion box for the ceramic inlet tube, allow the spring-loaded probe to contact the other end of the ceramic inlet tube. A small twist of the probe should allow the ceramic inlet tube to mate correctly with the probe. If the fit is poor the source may need to be moved slightly along the ion path to allow the probe to align correctly.
3. Using the pliers from the toolkit refit the connecting wires. Follow the table below to identify which lead to use for each connection. The connectors with the small holes fit on the source and the large holes fit on the feedthrough. Ensure that all wires do not touch and are clear of all other metal components.
4. Check that the connecting wires are attached correctly and that there are no short circuits. Use a meter from the feedthrough pins to the appropriate source component to check continuity.

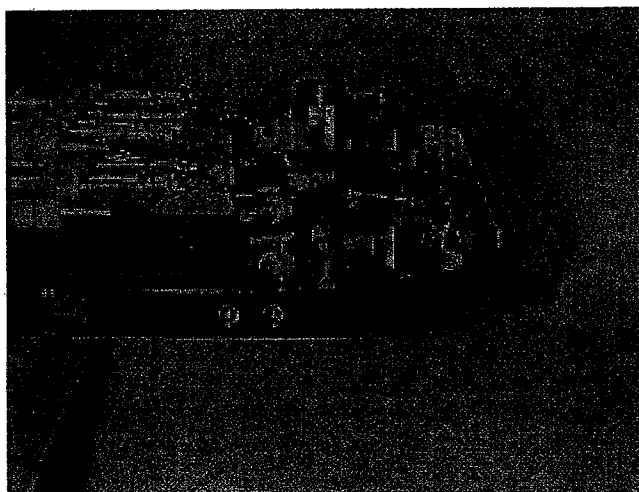


Photo of source connection points – right hand side

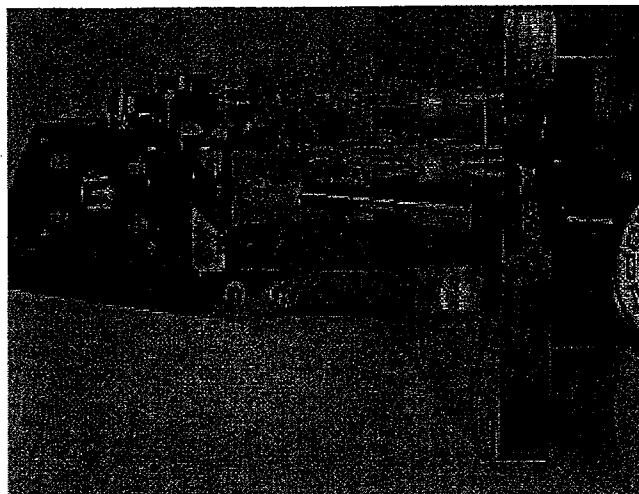
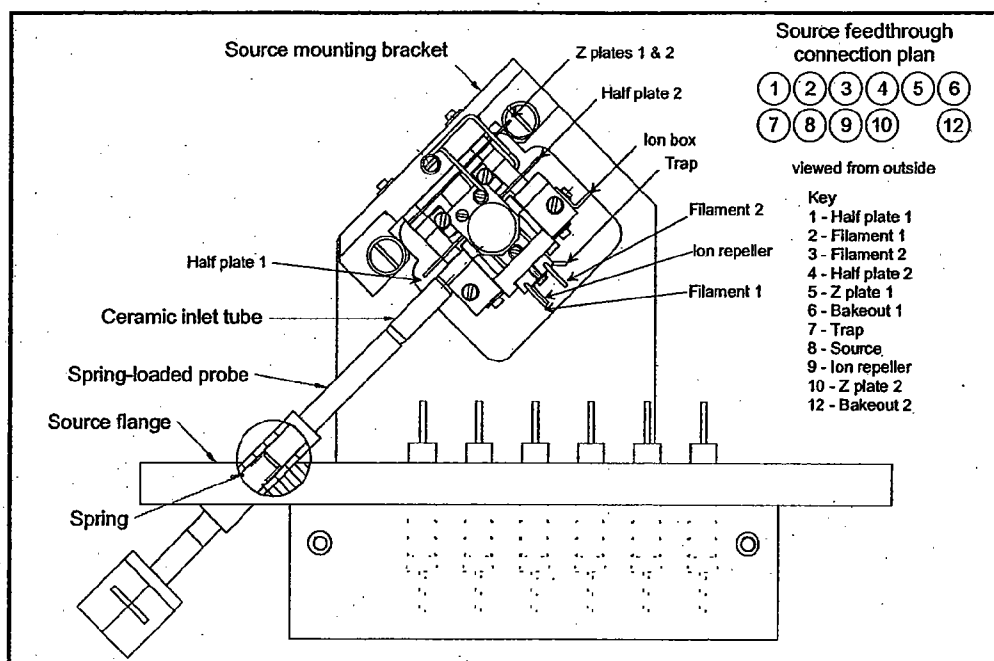


Photo of source connection points – left hand side

Table of source connection leads and connection points.

| Name | Length /mm | Source connection | Feedthrough pin | Type |
|------------------------|------------|-------------------|-----------------|-------------|
| Half plate 1 to flange | 70 | Half plate 1 | 1 | Kapton wire |
| Filament 1 to flange | 75 | Filament 1 | 2 | Bare copper |
| Filament 2 to flange | 65 | Filament 2 | 3 | Bare copper |
| Half plate 2 to flange | 95 | Half plate 2 | 4 | Kapton wire |
| Z1 to flange | 135 | Z plate 1 | 5 | Kapton wire |
| Trap to flange | 95 | Trap | 7 | Kapton wire |
| Ion repeller to flange | 60 | Ion repeller | 9 | Kapton wire |
| Ion box to flange | 100 | Ion box | 8 | Kapton wire |
| Z2 to flange | 135 | Z plate 2 | 10 | Kapton wire |



Source flange assembly drawing

Replacing the source flange



Caution: Cleaning solvents must not be used on Viton components as they may damage the Viton and be adsorbed. This results in poor vacuum whilst the Viton out-gasses.

Caution: Do not allow the sealing surface of the source flange to strike or rub the dowel pins. This will result in seal surface damage and a consequent vacuum leak.

The procedure for replacing the source flange is as follows:

1. Check that the sealing surfaces on the housing and the source flange are free of dust etc. If dust and fibres are adhering to the quad ring they may be removed by blowing with a supply of clean gas. A tissue soaked in acetone may be used to clean dirty sealing surfaces.
2. Offer the source flange to the housing. Connect the bakeout connections onto the feedthrough pins 6 & 12 (if fitted).
3. Align and engage the dowel pins with the holes in the source flange. Press the flange into position till it contacts the quad ring and hold in position.
4. Loosely replace the six bolts to hold the flange in position.
5. Tighten the mounting bolts in small amounts in the following order. Top centre, Bottom left, Top right, Bottom centre, Top left, Bottom right. Repeat this cycle until the quad ring is compressed and the flange contacts the analyser housing.
6. Reconnect the two plugs labelled J1 and J7 (J1 is closer to the source flange).
7. Reconnect the two half plate leads to pins 1 and 4.
8. Reconnect the two bakeout leads to pins 6 and 12.
9. Replace the cover and secure with the four screws.



Caution: Do not over-tighten the mounting bolts – the seal will not improve! If a vacuum leak is present it is necessary to remove the flange and check for damage to the seal surfaces or dirt on the quad ring.

Changing the Ion Source Filament



Caution: Wear hand protection (gloves) for this operation to avoid contamination.

Filament removal

The procedure for changing the Ion source filament is as follows:

1. Vent the instrument and remove the source flange.
2. Use the pliers from the toolkit to remove the filament connections and the trap connection.
3. Use the jeweller's screwdriver from the toolkit to remove the screw holding the heat conduction wire onto the top magnet assembly. Fold the wire out of the way of the magnet.
4. Undo the two screws that attach the top magnet holder to the defining slit holder and remove the magnet.
5. Undo the two screws that secure the filament support to the source block and remove the support and filament assembly.
6. Loosen the filament retaining screw from the support assembly, and remove the old filament.

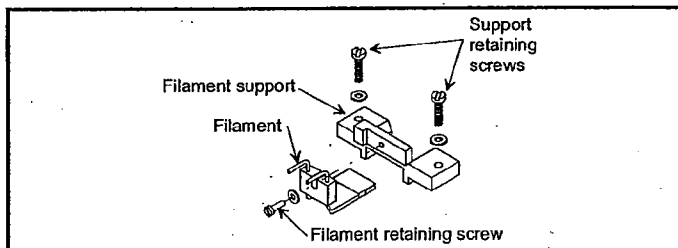


Diagram of filament support assembly

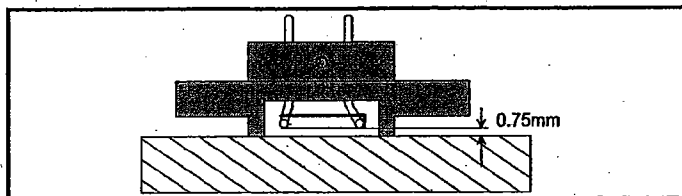
Filament positioning



The procedure for positioning the Ion source filament is as follows:

Caution: The filament coating is very fragile. Under no circumstances should the coating be touched or the filament wire distorted in any way.

1. Before attaching the new filament, make sure that the filament wire is securely attached to the posts, and has no kinks or other defects. The coating should be smooth and even with no imperfections along the centre of the filament and the shield does not touch both of the filament legs.
2. Secure the new filament to the support assembly with the filament retaining screw. Ensure that the filament retaining screw is seated in the bottom of the slot on the filament.
3. Place the magnet/filament assembly on a flat metal surface.
4. Adjust the filament so that the wire is parallel to the metal surface by twisting it around the filament retaining screw.
5. Adjust the height of the filament wire to approximately 0.75mm from the flat surface. Once the filament wire is parallel to the flat surface and at the correct distance away, lock the filament in place by tightening the retaining screw.

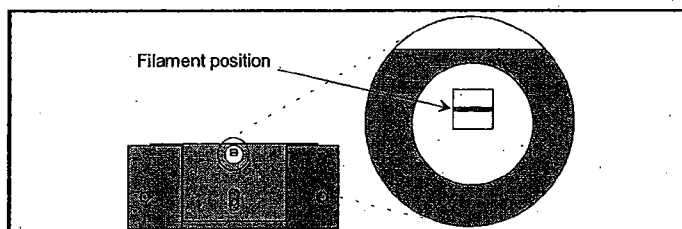


Setting the height of the filament

Filament assembly

The procedure for assembling the Ion source filament to the source is as follows:

1. Use the jeweller's screwdriver from the toolkit to remove the screw holding the heat conduction wire onto the bottom magnet assembly. Fold the wire out of the way of the magnet.
2. Undo the two screws that attach the bottom magnet holder to the defining slit holder and remove the magnet
3. Undo the two screws that secure the trap support to the source block and remove the support and trap assembly. Note: The trap ceramic is loose and may fall out.
4. Attach the filament support assembly to the source. Tighten the two retaining screws evenly so that the assembly is parallel to the source block.
5. Looking up through the trap aperture in the source wraparound, observe the filament position in the electron entrance aperture. The filament should lie parallel to the front face of the ion box (ion exit slit face) and be positioned at the mid-point of the electron entrance aperture.
6. Tighten the two filament support retaining screws evenly and re-check the filament position.
7. Attach the trap support assembly to the source. Tighten the two retaining screws evenly so that the assembly is parallel to the source block.
8. Check that the trap ceramic is correctly positioned at the end of the trap. Check that the trap, trap ceramic and source wraparound fit correctly and no gaps are visible.
9. Use an electrical meter to check for filament continuity and for any short circuit between the filament and the source block.
10. Use the pliers from the toolkit to replace the filament connections and the trap connection.
11. The source flange is now ready for replacement in the analyser housing.



Filament position within electron entrance aperture

Disassembly of the Ion Source



Caution: Wear hand protection (gloves) for this operation to avoid contamination.

The procedure for disassembling the Ion Source is as follows:

Note: All items referred to relate to the numbered parts in the ion source assembly diagram.



Caution: The ion source is a complex precision engineered assembly. It contains over 100 components. Incorrect assembly will have a serious effect upon its performance. For ease of re-assembly it is strongly recommended that the components be laid out in the order that they are disassembled. The user may wish to make additional notes in the manual during the procedure for clarification of certain points.

Magnet, filament and trap removal

1. Vent the instrument and remove the source flange.
2. Remove the ion source from the source flange.
3. Place the ion source on a flat clean surface, with the source mounting bracket (item 1) down.
4. Use the jeweller's screwdriver from the toolkit to remove the screw (item 40) holding the heat conduction wire onto the top magnet assembly. Fold the wire out of the way of the magnet.
5. Undo the two screws (item 26) that attach the top magnet holder to the defining slit holder and remove the magnet assembly.
6. Remove the screw (item 40) holding the heat conduction wire onto the bottom magnet assembly. Fold the wire out of the way of the magnet.
7. Undo the two screws (item 26) that attach the bottom magnet holder to the defining slit holder and remove the magnet assembly.
8. Undo the two screws (item 43) that secure the filament support to the source block and remove the support and filament assembly.
9. Undo the two screws (item 43) that secure the trap support to the source block and remove the support and trap assembly. Note: The trap ceramic is loose and may fall out.

Stack disassembly



Caution: The source assembly jig must be used for both the assembly and disassembly operations on the source. Failure to use the jig will result in metal wipes along the ceramic rods, which will provide a conductive path and promote flashover. This may render the source inoperative.



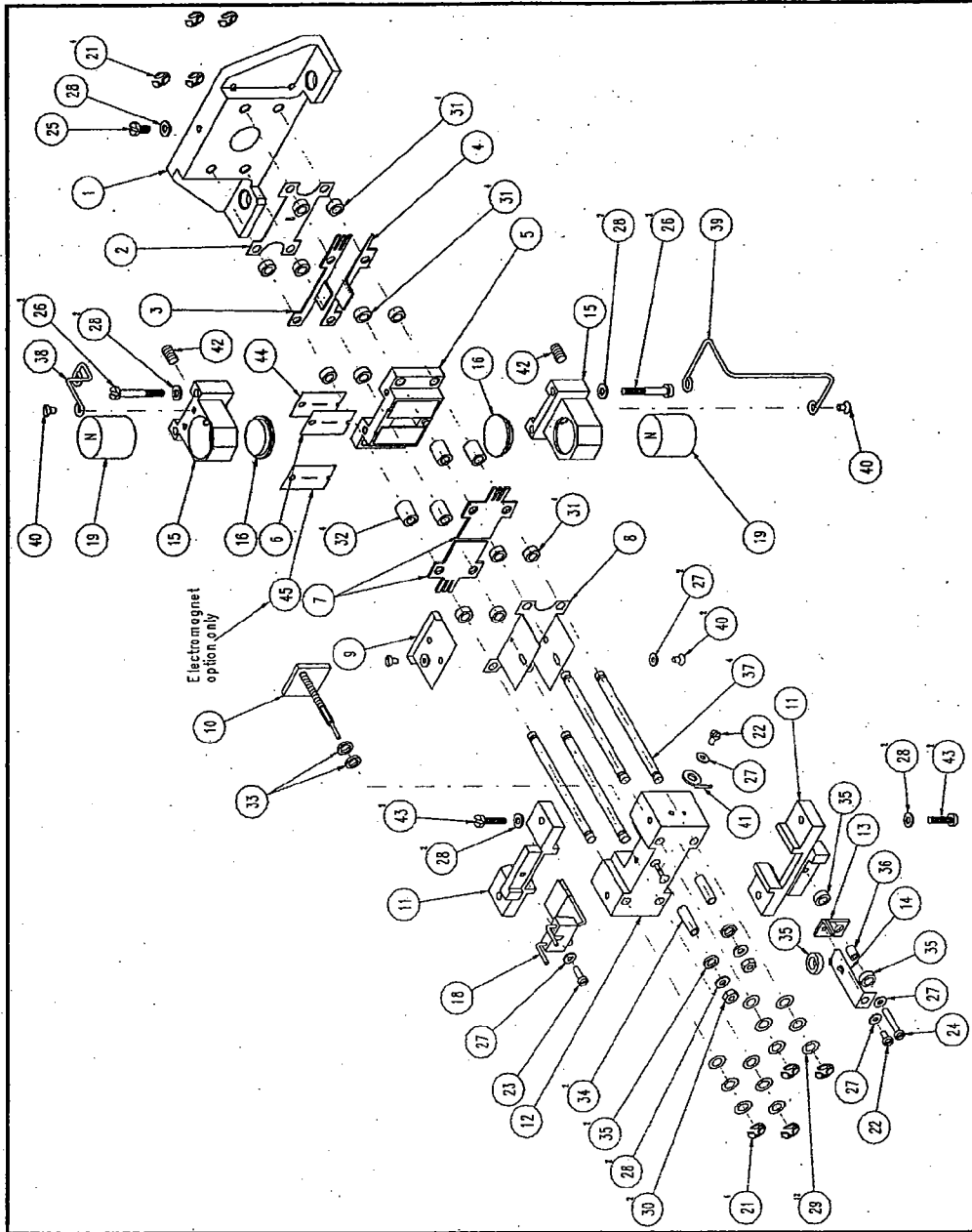
Caution: Ceramics are fragile and may be easily broken. Use caution and do not subject the ceramics to any shearing or twisting forces.

The procedure for disassembling the stack is as follows:

1. Rest the source on the mounting bracket so that the ion box is uppermost. Using the pliers from the toolkit, remove the circlips (item 21) from the ceramic rods (item 37).
2. Lift off the wavy washers (item 29), noting the number used.
3. Remove the ion box assembly from the ceramic rods.
4. Remove the four ceramic spacers (item 31).
5. Lift off the two half plates (item 7).

IsoPrime-EA User Manual

6. Fit the source assembly jig over the ceramic rods so that the protruding ceramic tubes of the jig contact the ceramic spacers (item 32).
7. Invert the source and jig so that the source mounting bracket is uppermost.
8. Remove the circlips from the ceramic rods.
9. Lift off the source mounting bracket. Note: Use care to ensure that the ceramic rods do not lift out when removing components from the source assembly.
10. Remove the alpha plate (item 2).
11. Lift off the four ceramic spacers (item 31).
12. Remove the z plates (items 3 & 4).
13. Lift off the four ceramic spacers (item 31).
14. Remove the source slit support (item 5).
15. Lift off the four ceramic spacers (item 32).
16. Remove the four ceramic rods (item 37) from the assembly jig.
17. Remove the source defining slit and support plate (if fitted) (items 6&44 or item 45) from the source slit support by sliding them out of the holder.



Ion source assembly diagram

IsoPrime-EA User Manual

Source Assembly Parts List

| Item no. | Qty. PM | Qty. EM | Item description | Part no. |
|----------|---------|---------|---|------------|
| 1 | 1 | 1 | BRACKET SOURCE MOUNTING | M693021CD1 |
| 2 | 1 | 1 | PLATE ALPHA GROUNDED 3mm | M693045BD1 |
| 3 | 1 | 1 | PLATE Z UPPER | M693023BD1 |
| 4 | 1 | 1 | PLATE Z LOWER | M693024BD1 |
| 5 | 1 | 1 | ASSEMBLY SLIT SUPPORT | M693025AC1 |
| 6 | 1 | | SHIM DEFINING SLIT 0.128 SLIT | M693282BD1 |
| 7 | 2 | 2 | PLATE HALF | M693029CD1 |
| 8 | 1 | 1 | PLATE ION EXIT SLIT 5mm | M693030CD1 |
| 9 | 1 | 1 | PLATE ION EXIT SHIELD | M693031BD1 |
| 10 | 1 | 1 | PLATE ION REPELLER | M693032BD1 |
| 11 | 2 | 2 | BLOCK FILAMENT SUPPORT | M693033CD1 |
| 12 | 1 | 1 | ION BOX TAPERED ENTRY | M693034DD1 |
| 13 | 1 | 1 | BRACKET TRAP | M693035AD1 |
| 14 | 1 | 1 | TRAP | M693036BD1 |
| 15 | 2 | 2 | HOUSING MAGNET 2mm EXTENDED | M693070CD1 |
| 16 | 2 | 2 | POLE SHOE TYPE 1 | M693073AD1 |
| 17 | | | | |
| 18 | 1 | 1 | ASSEMBLY 90° LEG FILAMENT | M693285BC1 |
| 19 | 2 | 2 | MAGNET | 7028110 |
| 20 | | | | |
| 21 | 8 | 8 | CIRCLIP TRUAC 5133/12H | T1026022 |
| 22 | 2 | 2 | SCREW CH HD M1.6x3 SS | 5314041 |
| 23 | 1 | 1 | SCREW CH HD M1.6x5 SS | 5314042 |
| 24 | 1 | 1 | SCREW CH HD M1.6x10 SS | 5314045 |
| 25 | 1 | 1 | SCREW CH HD M2 x 5 SS | 5314049 |
| 26 | 4 | 4 | SCREW CH HD M2 x 16 ST STL | 5314053 |
| 27 | 6 | 6 | WASHER FLAT M1.6 SS | 5331013 |
| 28 | 11 | 11 | WASHER FLAT M2 SS | 5331014 |
| 29 | 12 | 12 | WASHER WAVEY M3 SS | 5335005 |
| 30 | 2 | 2 | NUT M2 SS | 5321019 |
| 31 | 12 | 12 | CERAMIC SPACER 4.7 O/D x 2.9 I/D x 2 TH | 7020302 |
| 32 | 4 | 4 | CERAMIC SPACER 4.7 O/D x 2.9 I/D x 6 TH | S100009AD9 |
| 33 | 2 | 2 | CERAMIC SPACER 4.75 OD x 3.15 ID x 0.787 TH | T1636054 |
| 34 | 2 | 2 | CERAMIC INSULATOR 3 OD x 2 ID x 9 LG | M693072AD1 |
| 35 | 5 | 5 | CERAMIC SPACER 6.2 OD x 3.68 ID x 1.78 TH (XDA-084) | T1640052 |
| 36 | 1 | 1 | CERAMIC SPACER 3 OD x 2 ID x 6.4 TH | T1640016 |
| 37 | 4 | 4 | CERAMIC ROD \varnothing 2.75 x 45 LG | S100105AD8 |
| 38 | 1 | 1 | COOLING WIRE UPPER | M693162BD1 |
| 39 | 1 | 1 | COOLING WIRE LOWER | M693163BD1 |
| 40 | 2 | 2 | SCREW CSK M1.6 x 3 LG ST STL | 5311037 |
| 41 | 1 | 1 | TERMINAL WASHER | M702807AD1 |
| 42 | 2 | 2 | SCREW GRUB M3 x 4 SS | 5316005 |
| 43 | 4 | 4 | SCREW CH HD M2 x 8 SS | 5314065 |
| 44 | 1 | | DEFINING SHIM SUPPORT PLATE | M693291BD1 |
| 45 | | 1 | SHIM DEFINING SLIT 5mm | M693028BD1 |

Note: The columns Qty. PM and Qty. EM refer to the number of each item used within the ion sources for permanent magnet and electromagnet instruments respectively.

Disassembly of the ion box

The procedure for disassembling the ion box is as follows:

1. Remove the screw and washer (items 40 & 27) holding the filament shield (item 9) in position. Remove the filament shield.
2. Remove the screw and washer (items 40 & 27) on the other side of the wrap around (item 8). Remove the wrap around from the ion box.
3. Undo and remove the two set of nuts and washers (items 30 & 28) that retain the ion repeller in the ion box.
4. Lift off the ceramic (item 35).
5. Remove the ion repeller from the inside of the ion box.
6. Lift off the ceramics (items 35 & 34) from the ion repeller legs. Note: On some sources the ceramic (item 34) may be in two pieces.
7. If required, remove the bolt and screw (items 22 & 27) securing the terminal washer (item 41) to the source.

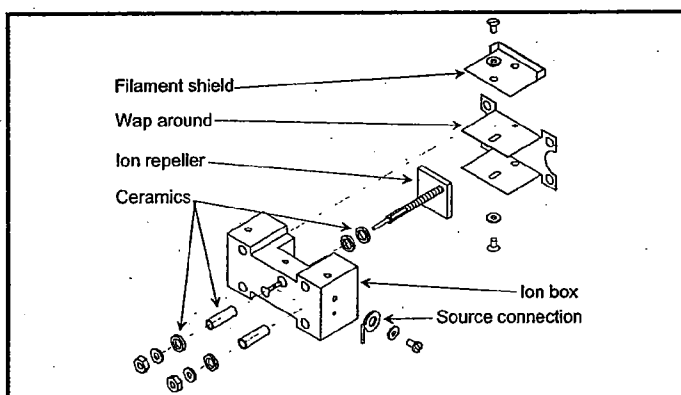


Diagram of ion box assembly

Dismantling the trap assembly

The procedure for dismantling the trap assembly is as follows:

1. Remove the trap ceramic from the trap (item 35) if it has remained in position.
2. Remove the screw and washer (items 22 & 27) that hold the trap to the trap bracket and remove the trap.
3. Remove the screw and washer that hold the trap bracket to the support block. Lift off the ceramics (items 35 & 36).

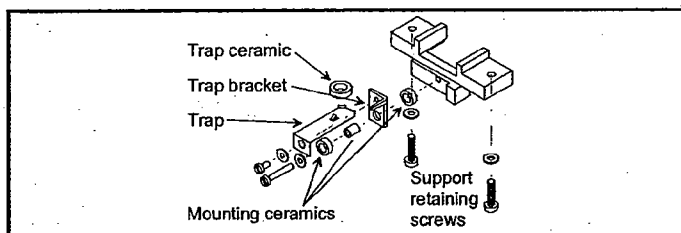


Diagram of trap assembly

Dismantling the magnet assembly



Caution: Magnets are brittle. Do not allow magnets to come together or contact magnetic materials under their own attraction. This may result in chipped or broken magnets. Damaged magnets will affect the operation of the source.

The procedure for disassembling the magnet assembly is as follows:

1. Prise the pole shoe from the end of the assembly.
2. Remove the grub screw (item 42) from the magnet housing.
3. Remove the magnet slug from the housing. Note the magnet orientation.

Assembly of the Ion Source



Caution: Wear hand protection (gloves) for this operation to avoid contamination.

The assembly of the ion source is largely the reverse of disassembly. Once assembled the source should be checked using a continuity to ensure that all plates are isolated and that the filament is not shorting. If available a Megger or other high voltage continuity tester should be used to check for shorts.

Folding photo-etched components

Because of the fragile nature of some of the components of the source it is impossible to supply them in a form ready for assembly. These photo-etched components are supplied flat for ease of packing and to limit the possibility of damage. The procedures below describe how to fold new photo-etch into the form in which it is used within the source.

Trap

The procedure to fold a trap from new photo-etch is as follows:

1. Using the pliers from the toolkit fold the two ears on the trap by gripping the ears and folding them so that the etch line is on the inside of the fold. Make a 90° bend.
2. Push the central finger up from the trap. Do not push the finger too far. Use the trap ceramic (item 35) and bend the finger to allow the ceramic to fit and be held in position by the finger.
3. Using the pliers from the toolkit fold the tab at the end of the trap. Ensure a clean straight fold of 90° perpendicular to the long axis of the trap.

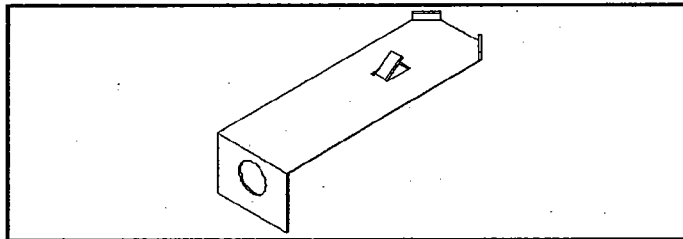


Diagram of folded trap

Source wrap-around

The procedure to fold a source wrap-around from new photo-etch is as follows:

Note: this procedure is best performed before starting any assembly of the source.

1. Using the back of the ion box as a pattern, sandwich the new photo-etch between the ion box and the side of the source assembly jig. Use two ceramic rods (item 37) inserted through the ion box as alignment guides. Ensure that the etched fold lines will be on the inside of the fold.
2. Position the first edge to be folded next to an edge of the assembly jig. Fold the side into position along the slot in the ion box. Using a clean stainless steel rod or the back of a scalpel, rub along the folded edge to ensure that it is folded correctly.
3. Rotate the ion box assembly and align the other fold with the edge of the assembly jig. Repeat the folding procedure as before.
4. Remove the ceramic rods and turn the ion box and wrap-around over. Check that the wrap-around is lying flat across the whole of the face of the ion box. Pay particular attention to the fold regions and look for any distortion across the corners where the ceramic rods fit.
5. Remove the wrap-around from the back of the ion box. The wrap-around is now ready for use.

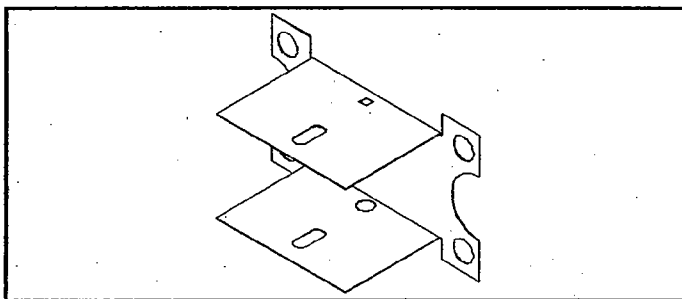


Diagram of folded source wrap-around

Filament shield

The procedure to fold a filament shield from new photo-etch is as follows:

1. Using the assembly jig, align the filament shield photo-etch on the assembly jig so that the long etch line lies along an edge.
2. Use a steel ruler to sandwich the filament shield onto the block.
3. Fold the edge of the filament shield over the assembly jig so that the etch line is on the inside.
4. Use a stainless steel rod or the back of a scalpel to rub the fold down and form a 90° bend.
5. Using the pliers from the toolkit fold the ears through 90° to form the filament shield as shown in the diagram below.

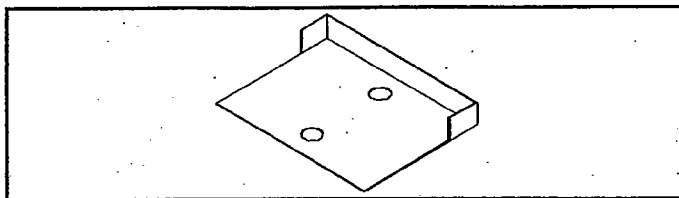


Diagram of folded filament shield

Reassembling the ion box assembly

The procedure for assembling the ion box assembly is as follows:

1. Refit the terminal washer (item 41) using the bolt and washer (items 22 & 27). Ensure that the tag points to the rear of the ion box as shown in the assembly diagram and protrudes at an angle of 45°.
2. Place the ceramics (items 35 & 34) onto each leg of the ion repeller. Ensure that item 34 fits within item 35.
3. Place the ion repeller in the ion box.
4. Place the ceramics (item 35) over the ion box legs and ceramics (item 34).
5. Fit the washers (item 35) and nuts (item 30) to the legs of the ion repeller.
6. Tighten the nuts carefully. Note: Over-tightening the nuts may break the ceramics.
7. Fit the wrap-around oriented so that with the tapered hole for the inlet probe is on the left of the ion box, the legs of the ion repeller are facing you the electron entrance hole (square) is on the top of the ion box.
8. Check that the wrap-around is lying flat against the front face of the ion box.
9. Fit the two screws and washers items 40 & 27) that hold the source wrap-around in position till they hold the wrap-around loosely in position.
10. The ion box assembly is now ready for assembly into the source stack.

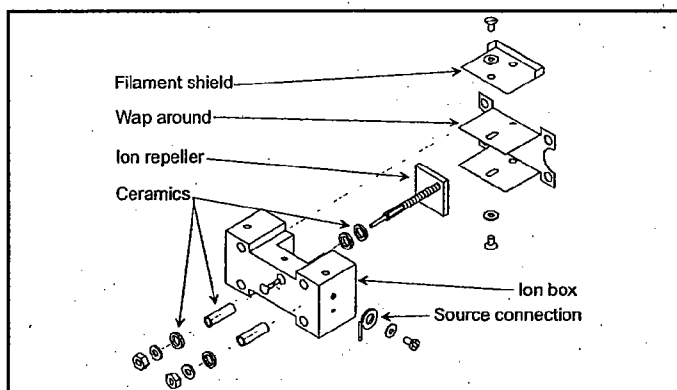


Diagram of ion box assembly

Reassembling the trap assembly

The procedure for assembling the trap assembly is as follows:

1. Mount the trap bracket on the support using the ceramics (items 35x2 & 36) and the mounting screw and washer (items 24 & 27).
2. Fit the trap to the trap bracket using the bolt and washer (items 22 & 27).
3. Fit the trap ceramic (item 35) to the trap and if required adjust the central finger of the trap to hold the ceramic in position.

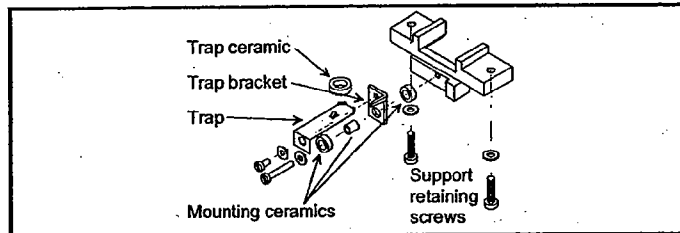


Diagram of trap assembly

Reassembling the magnet assembly



Caution: Magnets are brittle. Do not allow magnets to come together or contact magnetic materials under their own attraction. This may result in chipped or broken magnets. Damaged magnets will affect the operation of the source.

The procedure for assembling the magnet assembly is as follows:

1. Insert the pole face (item 16) into the magnet housing (item 15) and support so that the pole face lies flat against the face of the housing.
2. Insert the magnet slug (item 19) in the correct orientation (shown on the drawing) till it contacts the pole face.
3. Secure the magnet using the grub screw (item 42). Note do not over-tighten the screw or the magnet may be damaged.

Reassembling the stack



Caution: The source assembly jig must be used for both the assembly and disassembly operations on the source. Failure to use the jig will result in metal wipes along the ceramic rods, which will provide a conductive path and promote flashover. This may render the source inoperative.



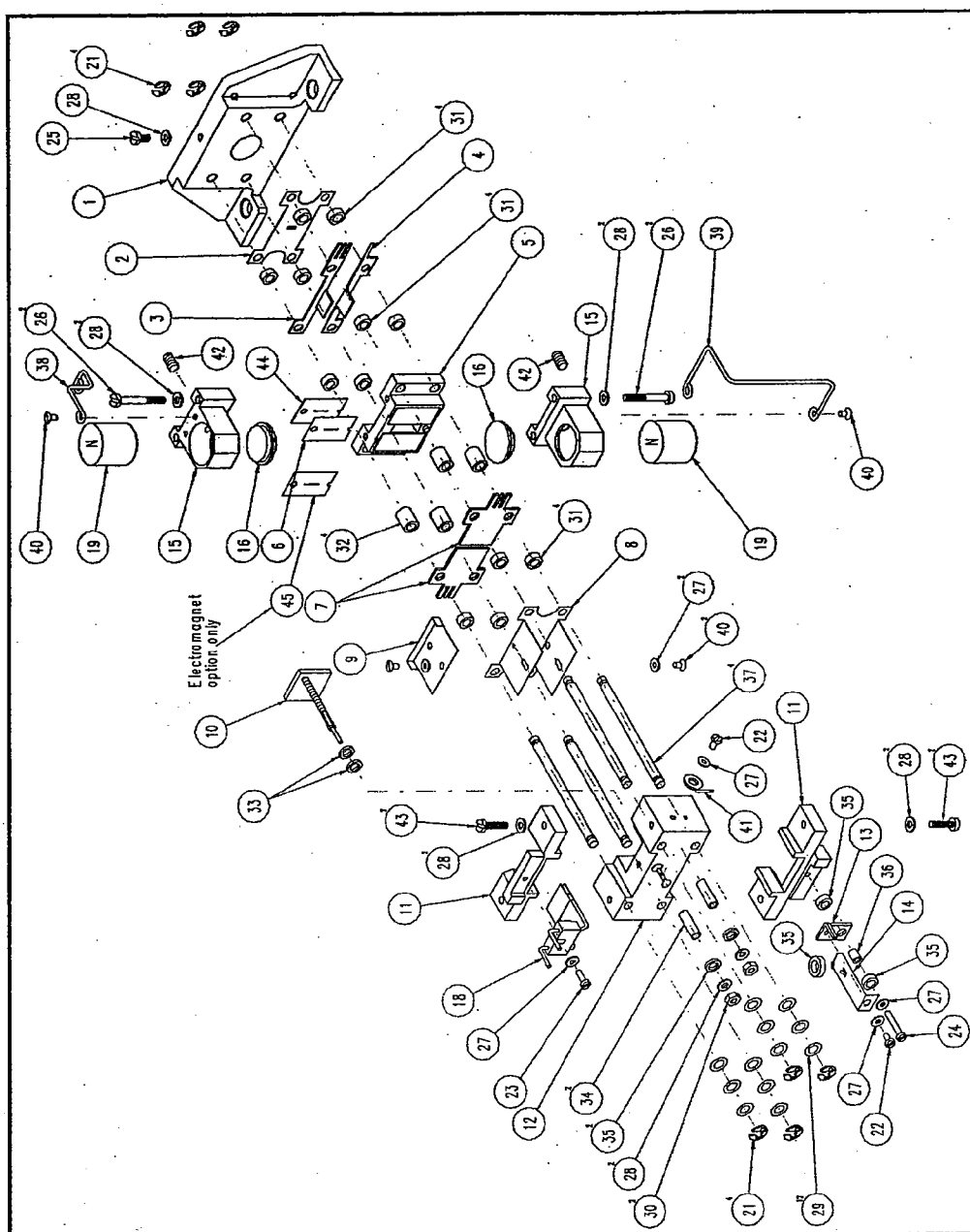
Caution: Ceramics are fragile and may be easily broken. Use caution and do not subject the ceramics to any shearing or twisting forces.

The procedure for reassembling the stack is as follows:

1. Place the assembly jig on the bench with a long side facing you and with the protruding ceramics uppermost. The face nearest you will become the trap side of the source (bottom) and the opposite face the filament side (top). The source is assembled from the middle to the ends to reduce the chance of metal wipes on the ceramics. The front of the source will be assembled first (toward the source mounting bracket).
2. Place the four ceramic rods (item 37) in the assembly jig.
3. Place the four ceramic spacers (item 32) on the rods.

IsoPrime-EA User Manual

4. Insert the source defining slit and support plate (if fitted) (items 6 & 44 or item 45) to the source slit support by sliding them into the holder. Note: The defining slit (item 6) should be closest the retaining plates of the support. The etched numbers on the defining slits should be visible. The slits should engage fully on the dowel in the support.
5. Replace the source slit support (item 5). Orient the support so that the raised section points down between the ceramics (item 32) and so that the dowel in the source slit support is closest you (the bottom).
6. Replace the four ceramic spacers (item 31).
7. Replace the z plates (items 3 & 4). Item 4 (z plate with only one finger) fits on the bottom two ceramic rods with the tab towards the centre of the source stack and pointing down. Item 3 (z plate with three fingers) fits on the top two ceramic rods with its tab facing the other z plate and pointing down.
8. Replace the four ceramic spacers (item 31).
9. Replace the alpha plate (item 2).
10. Replace the source mounting bracket.
11. Replace the circlips onto the ceramic rods.
12. Invert the source and jig so that the assembly jig is uppermost. Note: Do not allow the ceramic rods to slide out of the stack during the inversion process.
13. Remove source assembly jig from the ceramic rods.
14. Replace the two half plates (item 7).
15. Replace the four ceramic spacers (item 31).
16. Replace the ion box assembly on the ceramic rods.
17. Replace the wavy washers (item 29), using the number used previously.
18. Compress the source stack downwards and fit the circlips on the ceramic rods. It may be necessary to adjust the number of wavy washers if new components have been used in the assembly. The source stack should be held firmly together from the tension of the wavy washers on the circlips.
19. Ensure that the source wrap-around is fitted snugly to the ion box and tighten the retaining screws (items 40 & 27).
20. The stack assembly is now complete.
21. Refit the other source components as detailed under 'Changing the ion source filament'.



Ion source assembly diagram

IsoPrime-EA User Manual

Source Assembly Parts List

| Item no. | Qty. PM | Qty. EM | Item description | Part no. |
|----------|---------|---------|---|------------|
| 1 | 1 | 1 | BRACKET SOURCE MOUNTING | M693021CD1 |
| 2 | 1 | 1 | PLATE ALPHA GROUNDED 3mm | M693045BD1 |
| 3 | 1 | 1 | PLATE Z UPPER | M693023BD1 |
| 4 | 1 | 1 | PLATE Z LOWER | M693024BD1 |
| 5 | 1 | 1 | ASSEMBLY SLIT SUPPORT | M693025AC1 |
| 6 | 1 | | SHIM DEFINING SLIT 0.128 SLIT | M693282BD1 |
| 7 | 2 | 2 | PLATE HALF | M693029CD1 |
| 8 | 1 | 1 | PLATE ION EXIT SLIT 5mm | M693030CD1 |
| 9 | 1 | 1 | PLATE ION EXIT SHIELD | M693031BD1 |
| 10 | 1 | 1 | PLATE ION REPELLER | M693032BD1 |
| 11 | 2 | 2 | BLOCK FILAMENT SUPPORT | M693033CD1 |
| 12 | 1 | 1 | ION BOX TAPERED ENTRY | M693034DD1 |
| 13 | 1 | 1 | BRACKET TRAP | M693035AD1 |
| 14 | 1 | 1 | TRAP | M693036BD1 |
| 15 | 2 | 2 | HOUSING MAGNET 2mm EXTENDED | M693070CD1 |
| 16 | 2 | 2 | POLE SHOE TYPE 1 | M693073AD1 |
| 17 | | | | |
| 18 | 1 | 1 | ASSEMBLY 90° LEG FILAMENT | M693285BC1 |
| 19 | 2 | 2 | MAGNET. | 7028110 |
| 20 | | | | |
| 21 | 8 | 8 | CIRCLIP TRUAC 5133/12H | T1026022 |
| 22 | 2 | 2 | SCREW CH HD M1.6x3 SS | 5314041 |
| 23 | 1 | 1 | SCREW CH HD M1.6x5 SS | 5314042 |
| 24 | 1 | 1 | SCREW CH HD M1.6x10 SS | 5314045 |
| 25 | 1 | 1 | SCREW CH HD M2 x 5 SS | 5314049 |
| 26 | 4 | 4 | SCREW CH HD M2 x 16 ST STL | 5314053 |
| 27 | 6 | 6 | WASHER FLAT M1.6 SS | 5331013 |
| 28 | 11 | 11 | WASHER FLAT M2 SS | 5331014 |
| 29 | 12 | 12 | WASHER WAVEY M3 SS | 5335005 |
| 30 | 2 | 2 | NUT M2 SS | 5321019 |
| 31 | 12 | 12 | CERAMIC SPACER 4.7 O/D x 2.9 I/D x 2 TH | 7020302 |
| 32 | 4 | 4 | CERAMIC SPACER 4.7 O/D x 2.9 I/D x 6 TH | S100009AD9 |
| 33 | 2 | 2 | CERAMIC SPACER 4.75 OD x 3.15 ID x 0.787 TH | T1636054 |
| 34 | 2 | 2 | CERAMIC INSULATOR 3 OD x 2 ID x 9 LG | M693072AD1 |
| 35 | 5 | 5 | CERAMIC SPACER 6.2 OD x 3.68 ID x 1.78 TH (XDA-084) | T1640052 |
| 36 | 1 | 1 | CERAMIC SPACER 3 OD x 2 ID x 6.4 TH | T1640016 |
| 37 | 4 | 4 | CERAMIC ROD ø 2.75 x 45 LG | S100105AD8 |
| 38 | 1 | 1 | COOLING WIRE UPPER | M693162BD1 |
| 39 | 1 | 1 | COOLING WIRE LOWER | M693163BD1 |
| 40 | 2 | 2 | SCREW CSK M1.6 x 3 LG ST STL | 5311037 |
| 41 | 1 | 1 | TERMINAL WASHER | M702807AD1 |
| 42 | 2 | 2 | SCREW GRUB M3 x 4 SS | 5316005 |
| 43 | 4 | 4 | SCREW CH HD M2 x 8 SS | 5314065 |
| 44 | 1 | | DEFINING SHIM SUPPORT PLATE | M693291BD1 |
| 45 | | 1 | SHIM DEFINING SLIT 5mm | M693028BD1 |

Note: The columns Qty. PM and Qty. EM refer to the number of each item used within the ion sources for permanent magnet and electromagnet instruments respectively.



Penning Gauge Maintenance

Please refer to manufacturer's guide supplied and the cleaning procedure below.

Cleaning Procedures

This is a generalised cleaning procedure for both ceramic and stainless steel components used in the source. If more details are required then please contact Micromass UK Ltd Customer Service Department or the local representative.



Caution: Some of the components used within the IsoPrime are fragile and care must be taken when cleaning to avoid damage. Note: The filament should not be cleaned under any circumstances.



Warning: The cleaning procedures involve the use of boiling water and strong chemicals. It is important that the user assess the risks involved in the use of these materials before use.

Rough Cleaning

Polaris Powder (Al_2O_3 powder) or Scotchbrite can be used to remove obstinate marks (burn marks, etc.).

Note: When cleaning the ion repeller use metal polish to avoid scratching the surface. The ion repeller should have a mirror polish finish.

Pre-cleaning

For stainless steel components immerse in hot solvent cleaner in ultra-sonic for 30 minutes or hot ultra-sonic wash in Decon 90 (or similar aqueous cleaner) for 15 minutes followed by a rinse in de-ionised water. Either of these processes should remove any fingerprints and mild contamination.

For ceramic components immerse in boiling in Decon 90 (or similar aqueous cleaner) for 30 minutes followed by a rinse in de-ionised water.

Final Cleaning

This is a generic cleaning procedure for any component that can withstand the conditions. Exceptions include Nylon, O-rings, Kel-F, etc.

1. Immerse in a clean container with high purity Acetone. Place in an ultrasonic bath and leave to clean for 15 -20 minutes.
2. Boil in de-ionised water for 30 minutes.
3. Change the water and boil for a further 30 minutes
4. Dry in oven at 100 to 120° C for 12 hours.

After cleaning all components should only be handled using gloves.

If the flight tube requires cleaning, chemical cleaning alone is rarely sufficient.

Please, follow the procedure outlined below:

1. In the first instance, the flight tube needs to be scrubbed with a wire brush, paying particular attention to any burn marks at the entrance of the tube.
2. Wash off all the solid residues with plenty water.

IsoPrime-EA User Manual

3. Boil for 30 minutes in deionised water.
4. Rinse with high purity Acetone
5. Ultrasonic for 15-20 minutes in high purity Acetone.
6. Rinse once more with Acetone.
7. Dry in the oven at 120° C for 2 hours.

Penning gauge cleaning

1. Remove the electronic podule carefully.
2. Using a proper circlip tool, remove the circlip from the tube.
3. Carefully, pull out the cathode cup and the cathode plate.
4. Use an AIM spanner to undo the clamp collar from the other end of the tube.
5. Pull the anode assembly without twisting the anode.
6. Remove the O-ring.
7. Use an ultrasonic tank to clean all the above components with a proper diluted solvent for about 10 minutes. Repeat the process with alcohol for 5 minutes.
8. If necessary use a very fine emery paper to clean the gaps between the strikers and the shield disc.
9. Adjust the gap between the strikers and the shield disc by bending the striker (not the shield disc). The gaps between the striker and the shield disc should be maintained between 0.25 to 0.35 millimetres.
10. Re-assemble the electrodes in the tube in the same way as indicated in the AIM gauge's instruction manual.

Rotary Pump Repair

Please refer to manufacturers guide for details.

Turbomolecular Pump and Vent Valve Repair

Please refer to manufacturers guide for details.

Air Compressor (if supplied) Repair

Please refer to manufacturers guide for details.

Electronics Repair



Caution: Suitably qualified personnel only should perform maintenance operations. Please contact the Customer Service Department at Micromass or your local Micromass representative with any queries.

Removal of the system controller

1. The main electronics for the IsoPrime is located in the right hand side of the IsoPrime cubicle.
2. It is recommended that the IsoPrime be vented before replacing this unit otherwise the following steps must be completed within 5 minutes.

IsoPrime-EA User Manual

3. Remove IEC connector to the Electronics unit (and the IEC connector to The bakeout supply if fitted) and all connectors to the unit including the two 6W QM connectors underneath the HV shield at the top of the instrument. Disconnect the two half plate leads (and bakeout leads if fitted) from the analyser and push them back in the hole of the electronics box.
4. Undo the screw at the front of the electronics unit.
5. Pull handle to the left to clear the door and pull out.

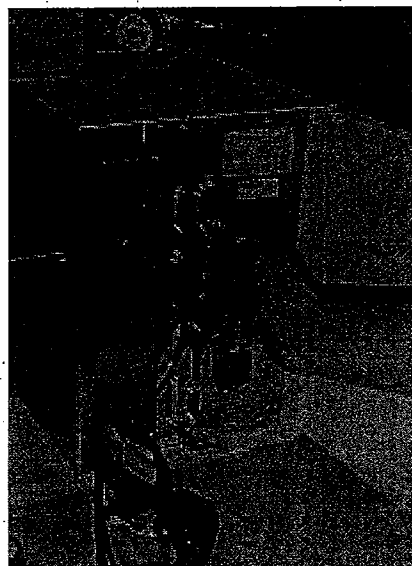


Photo of system controller removal (bakeout supply shown fitted)

Refitting the system controller

1. Ensure that the half plate and bakeout leads are pushed inside the electronics unit
2. Push in the electronics unit
3. Retrieve the half plate wires and bakeout cables from inside the unit and connect to the correct pins on the analyser (see label on the instrument). Refit the two 6W rectangular QM connectors ensuring that all the wires mate properly with the pins on the analyser
4. Refit the HV shield
5. Screw in the screw at the front of the electronics (note the box may have to be moved around until the screw locates in the hole)
6. Refit all cables except the IEC lead.
7. Note J2 is only used if an electromagnet is fitted.
8. J5 is used for interfaces requiring extra valves i.e. the Trace gas preconcentrator
9. J6 is the standard valve connector for controlling RG etc.
10. Refit the IEC plug.

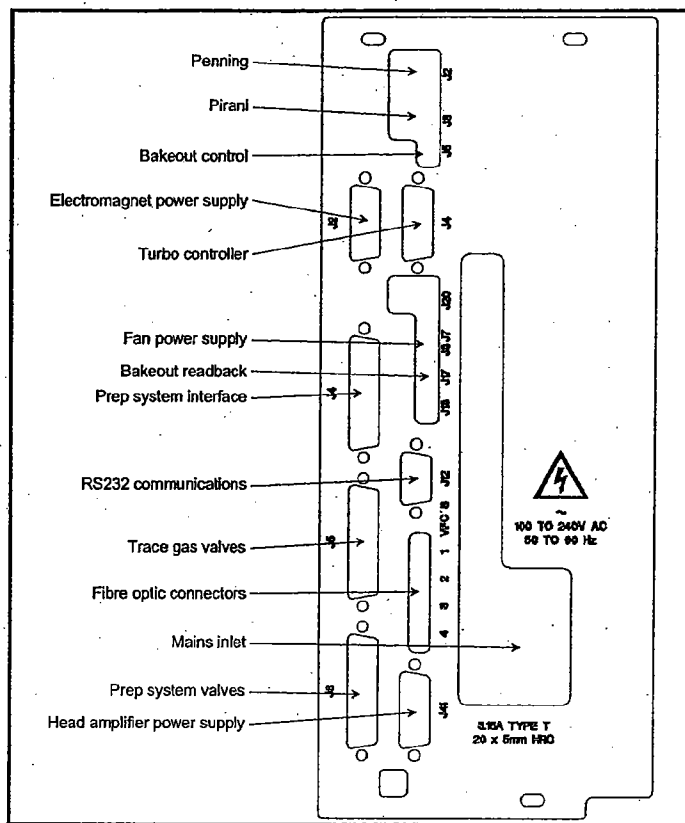


Diagram of system controller front panel layout

Removal of the bakeout power supply

1. Remove Electronics unit with bakeout unit attached as described in the previous section
2. Undo the 4 screws that hold the bakeout supply onto the electronics box.
3. Pull the two wires through the electronics box



Photo of removal of bakeout supply

Replacement of the bakeout power supply Ensure the blanking plug is replaced with the grommet (this will involve removal of the lid of the electronics box.

1. Ensure that the bakeout supply is set to the correct voltage.
2. Push the two wires through this hole
3. Screw the bakeout unit to the electronics box using the four screws
4. Replace the electronics unit into the IsoPrime as described in the previous section.

Removal of the electromagnet power supply

Remove the lower blue cover by pulling the top forward and then lifting the cover out of the mounting slots.

Remove IEC connector at the back of the electromagnet PSU

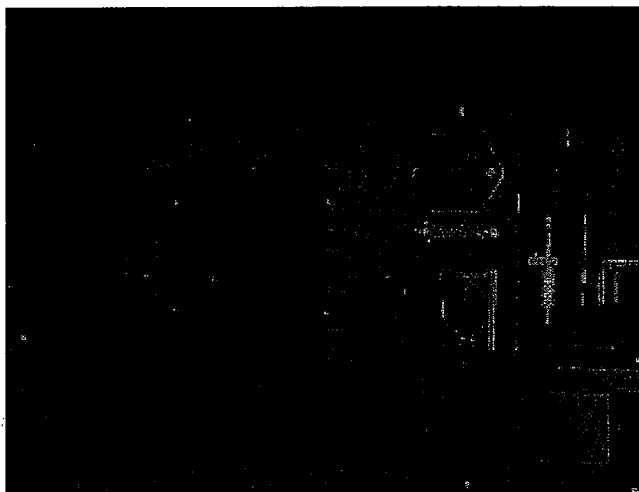


Photo of rear connections on electromagnet power supply

Remove the 4 screws that hold the power supply in position



Photo of electromagnet power supply mounting

IsoPrime-EA User Manual

Pull the power supply forward

Remove the 15W D connector

Remove XLR connector by pushing the button in and pulling the connector out

Replacement of the electromagnet power supply

1. Check the Voltage Selector is set for the correct supply
2. Refit the XLR Connector
3. Refit the 15W D Connector
4. Push unit back into the metalwork
5. Screw in the 4 screws
6. Reconnect the IEC mains connector

See setting up the Electronics in the using a terminal emulator section if this is a new addition to your IsoPrime

Removal of the head amplifier

1. Turn off source
2. Remove the 5 W Din connector
3. Remove The 4 fibre optic cables
4. Undo the two hex screws holding the unit on

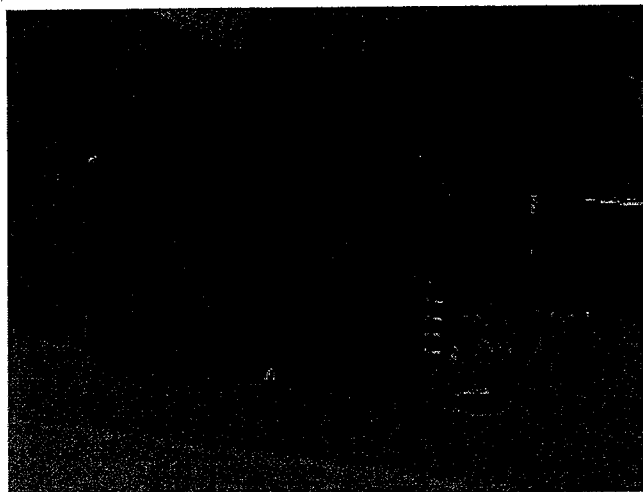


Photo of head amplifier removal

Replacement of the head amplifier

Using a suitable metal implement, short the feedthrough pins to the metal of the analyser



Photo of shorting of head amp feedthrough pins

Slide the head amp over the dowels

Screw up the two hex screws

Replace the 5W Din connector

Replace the fibre optic cables

Note From Top to Bottom they go in the order 3, 2, 1, 4. The very top one is not used.

IsoPrime user replaceable fuse information

Note: In all applications using two fuses, for maximum reliability replace **both** fuses if one fuse has blown.

Mains inlet to IsoPrime

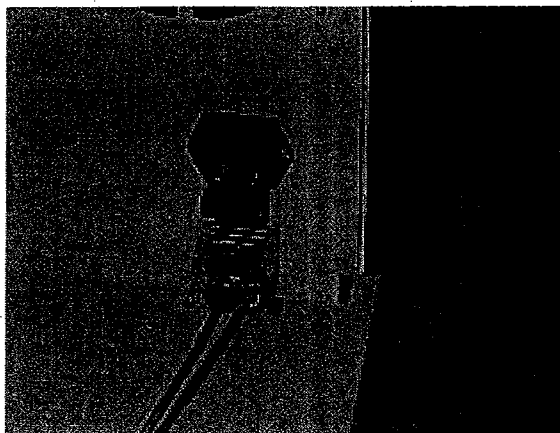


Photo of mains inlet fuses

2 x 10A T HRC 20mm 250V ceramic fuses part no. 1340205

System controller

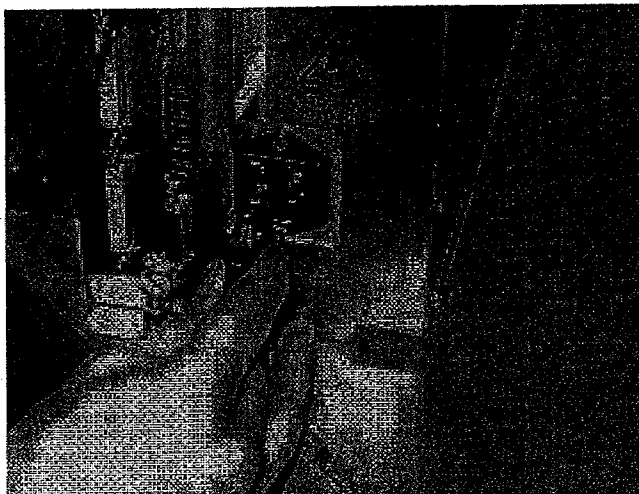


Photo of system controller fuses

2 x 3.15A T HRC 20mm 250V ceramic fuses part no. 1340217

Bakeout supply

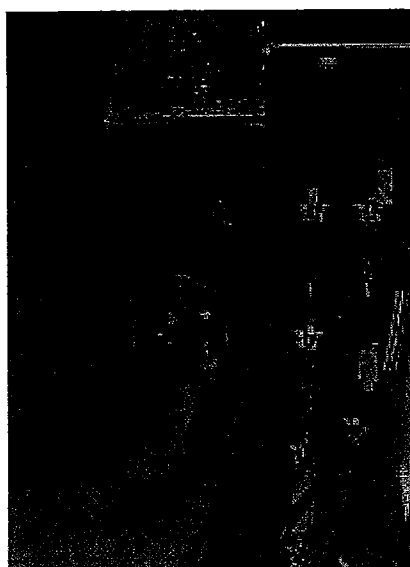


Photo of bakeout supply fuses

2 x 3.15A T HRC 20mm 250V ceramic fuses part no. 1340217

Electromagnet supply

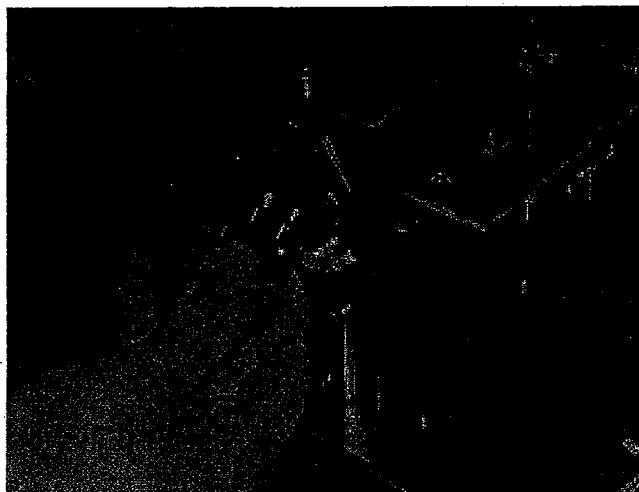


Photo of electromagnet power supply fuses

2 x 3.15A T HRC 20mm 250V ceramic fuses part no. 1340217

Computer, Monitor and Printer Repair

Please refer to manufacturers guide for details.

Routine Maintenance for the EA

Routine maintenance of the elemental analyser is generally associated with replacement of consumable such as oxidation, reduction, absorbent filter tubes, along with their relevant packing materials. In addition the build up of ash in the combustion tube needs to be controlled. Detailed aspects of these and other less frequent maintenance considerations are detailed in section 5 of the elemental analyser user manual.



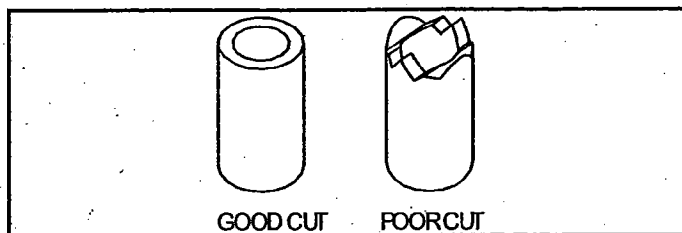
Caution: Before carrying out any operation which involves breaking the flow of helium to the mass spectrometer close the Nupro isolation valve to the mass spectrometer.

Cutting vitreous silica capillaries

Using the ceramic capillary cutter supplied with the toolkit, scratch the surface of the polyimide at right angles to the capillary. Ensure that the capillary does not break or smash at this stage, otherwise glass dust will result, thus forming active sites where the chromatography may degrade.

When the scratch has been made, bend the capillary gently, until it breaks off.

Cuts, performed in this manner, are usually clean. It is, however, advised to inspect the cut using a magnifying glass. Inspection at this stage takes only a few seconds, it could take a significant length of time to diagnose the problem site, later on, when the entire system has been assembled together.




Replacing the reference capillary

The reference line capillary should never require changing. However it may be broken accidentally in which case the following procedure should be carried out.

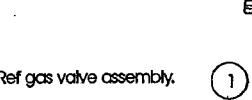
Note: This procedure assumes the sample line is connected to the splitter tee and the flow of sample line helium through the splitter into the mass spec registers between 2 and 4E-6mb.

1. Close the Nupro isolation valve.
2. Switch off the source.
3. Remove the old capillary from the 1/16th tee connecting it to the 1/16th by 0.020" stainless steel capillary leading to the Nupro valve.
4. Remove the old capillary from the reference open split line.
5. Cut 2 metres of 75um fused silica quartz capillary.
6. Push one end of the glass capillary through the tee into the stainless steel capillary leading to the Nupro valve a distance almost but not quite up to the Nupro valve itself.
7. Fix the capillary along with the one coming from the prep system to the tee with a 1/16th SSUT nut and GVF(2) 004 ferrule.
8. Engage the other end of the capillary a distance of 50cm minimum into the reference line open split.
9. Open the Nupro valve check the penning gauge reading is in the region of 2-4E-6mb. If it is much greater then suspect that the GVF 004 ferrule is not tight enough.
10. Switch on the source and check for leaks, initially monitoring the background nitrogen. A virtual leak such as out-gassing associated with the new capillary will soon decay
11. Open the reference gas valve and introduce CO₂. Tune the source to CO₂ and measure the transport time associated with the new capillary. This time should not differ greatly from the previous capillary.
12. Carry out reference gas stability tests on both CO₂ and N₂ until the system stabilises.

STEP 1. Fitting the vitreous silica capillary.



STEP 2. Ref gas valve assembly.

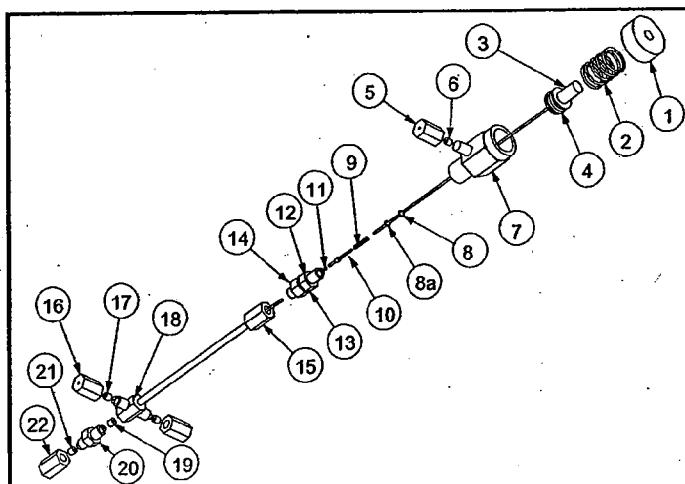


The reference gas assembly is re-built according to the diagram shown above. Additionally, take the following precautions:

- Ensure that the RG valve is OPEN whilst performing this operation, otherwise, damage may result to the valve needle.
- Ensure that the 25 μ m capillary (25 μ m x 350 μ x 7cm) does not protrude beyond the flat face of the GVF 003/004 ferrule. This ferrule has been especially drilled, to allow the capillary to engage into the conic section up to approximately 1mm from the flat face. Provided that the capillary has an OD greater than 0.3 mm, it will be impossible for it to pass through to the upper face.
- Ensure that the 25 μ m capillary is cut clean. Please inspect the cleanliness of the ends, using a magnifying glass.
- Ensure that the stainless steel capillary, labelled with the number 6 on the diagram above, is not engaged into the tee union, beyond the start of the tee inlet port. If this is allowed to happen, the flow of Helium gas will be adversely impeded at this site.
- Ensure that all the other stainless steel capillaries are mounted in their respective connectors with the minimum of dead volume. That is, they should all be butt mounted.
- Leak check the entire assembly thoroughly, after re-assembly has been completed. Please refer to the subsection titled Cleanliness of the vacuum and leak checking.

The MOVPT valve

This is a very robust valve, however, on rare occasions it is possible that it develops a leak between the pneumatic head and the valve body. A schematic of this valve, as supplied by SGE, the manufacturers of the valve, is included below.

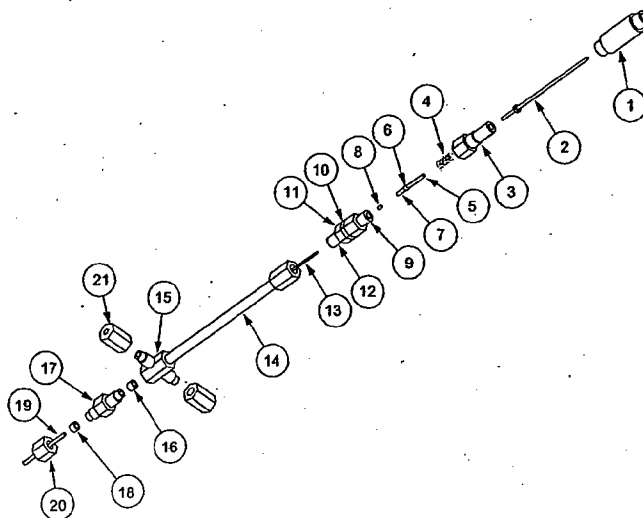


- | | |
|-------------------------|-----------------------|
| 1. Pneumatic Head Cap | 2. Piston spring |
| 3. Piston Plunger shaft | 4. Skirt seal |
| 5. BN16 nut | 6. GFF-16 ferrule |
| 7. Pneumatic body | 8. "O" ring seal |
| 8a. Flat MOVPT washer | 9. MVTL seal spring |
| 10. Thrust tube | 11. PTFE Shaft seal |
| 12. Brass union | 13. Star washer |
| 14. Lock nut | 15. Lower stem |
| 16. SN-16 nut | 17. VSR-16 ferrule |
| 18. Stem body | 19. Vespel valve seal |
| 20. SS union | 21. GFV004 ferrule |
| 22. SN-16 nut | |

The cause for the leak mentioned above is the PTFE shaft seal, designated by the item number 11. Please, replace this seal, according to the diagram supplied.

The MCVT-1 splitter valve

This valve is very robust in operation and will rarely need attention. However, it can develop a leak on the PTFE seal to atmosphere. A schematic of this valve, as supplied by SGE, gives a clear description of the replacement procedure.



- | | |
|----------------------|-----------------------|
| 1. Control knob | 2. Needle |
| 3. Upper assembly | 4. Compression spring |
| 5. Thrust tube | 6. Thrust tube stop |
| 7. Thrust tube | 8. PTFE seal |
| 9. Male thread | 10. Washer |
| 11. Locknut | 12. Union |
| 13. Needle | 14. Lower stem |
| 15. Valve body | 16. Valve seat |
| 17. SS Union | 18. ferrule |
| 19. 1/16th capillary | 20. Nut |
| 21. Nut | |

Changing Gas Bottles

Helium



Caution: The Nupro isolation valve must be closed whilst changing the Helium gas bottle. The EA TCD filament must be switched off and the bottle must be changed as quickly as possible to protect the EA combustion and reduction tubes.

After changing the Helium gas bottle, please, take the following precautions:

- Leak check the connections you have disturbed at the gas supply bottle regulator, using Snoop, or some other leak checking solution.
- Check that the gas supply pressure at the bottle is set to 4 bar.
- Allow all the gas lines to purge for several minutes, prior to re-opening the isolation valve.
- Take all the necessary precautions, as the isolation valve is re-opened as described in section 6, the section on setting up the reference gas injector.

Reference gas

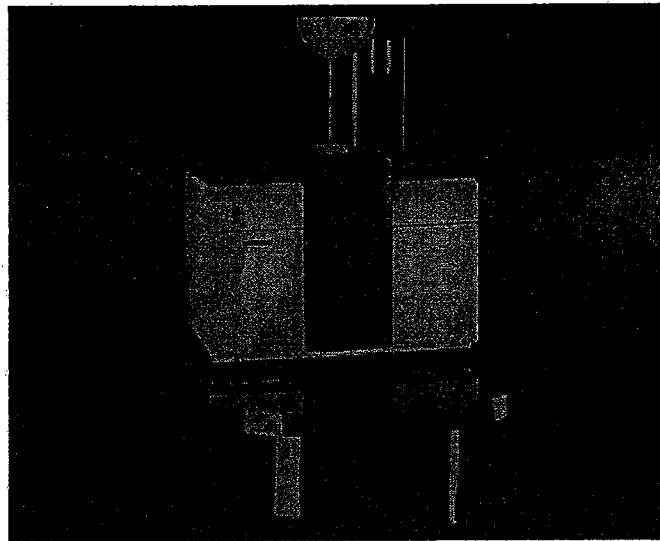
Unlike the replacement of Helium, it is not necessary to close the isolation valve, whilst changing the reference gas bottles.

After the bottle has been changed, the entire gas lines must be purged to eliminate all traces of atmospheric gases. This is best achieved by opening fully the needle valve that controls the flow of gas at the reference gas purge outlet.

Leave the needle valve fully open for a few minutes, then reduce the flow to 5 ml min⁻¹.

IsoPrime-EA User Manual

Section 9



Fault Finding

IsoPrime-EA User Manual

| | |
|---|----------|
| Fault finding | 5 |
| I cannot achieve the recommended operating pressures | 5 |
| On the Pirani gauge | 5 |
| On the Penning gauge | 5 |
| I cannot achieve the recommended background levels | 6 |
| For Argon | 6 |
| CO2 and N2 | 6 |
| The background scan is dirty | 6 |
| I cannot operate the valves | 7 |
| I cannot get a good mass spec peak shape | 7 |
| I cannot get stability | 7 |
| The reference gas pulses have an abnormal shape | 7 |
| The reference gas pulses have a normal shape | 8 |
| I cannot get linearity | 8 |
| I cannot get precision on the EA | 9 |
| I cannot get accuracy | 9 |
| The inaccuracy is constant across a variety of standards | 9 |
| The inaccuracy is random across a range of standards | 9 |
| I have poor sensitivity | 10 |
| Sample sensitivity is poor, reference gas sensitivity is OK | 10 |
| Both sample and reference gas sensitivities are poor | 10 |
| Sample sensitivity good, Reference sensitivity poor | 10 |
| I have no sample peak | 11 |
| No sample peak on MS and no signal when RG is open | 11 |
| No sample peaks on MS, Ms OK, no sample peak on TCD | 11 |
| No sample peak at MS, MS OK, sample peak at TCD OK | 11 |
| Sample peak at MS OK, no sample peak on the TCD | 11 |
| I have poor chromatography | 11 |
| On both the TCD and the mass spectrometer | 11 |
| On the mass spectrometer alone | 12 |
| System Controller Error Messages | 12 |
| Electronics | 14 |
| System Crashes | 14 |
| Problems Controlling the Source/HT Units | 14 |
| Magnet Supply Problems (where applicable) | 14 |
| Beam Problems | 14 |
| Fault Diagnosis Using a Terminal Emulator | 14 |

IsoPrime-EA User Manual

| | |
|---------------------------------------|-----------|
| General Notes on Fault Finding..... | 16 |
| Contact details | 16 |
| Practical considerations | 16 |
| Using the micro balance. | 17 |
| Managing memory..... | 19 |

Fault finding

This section has been included in order to help the user diagnose the source of a fault, should a problem be encountered. It can only be expected to serve as a guide rather than a complete description of all situations that may arise as the instrument is used. It may be worthwhile to keep a record of any experiences, if it is felt that any information gathered in this way is of general interest and should be included in future versions of this manual, please, contact the customer service department at Micromass UK Ltd.

I cannot achieve the recommended operating pressures

On the Pirani gauge

The normal operating reading on the Pirani gauge is 10^{-3} mbar. After breaking vacuum it may take some time to reach this pressure, in particular if the atmospheric moisture levels were high at the time that the analyser was open to atmosphere. It could take one day to return to 10^{-3} mbar. If this cannot be achieved, then:

- Check for leaks with Acetone.

Caution: Never use Acetone on a Viton seal. Never use water to leak check.

If Acetone is applied at a leaking site, at first, the Pirani reading may decrease. The liquid Acetone, in this instance forms a temporary "plug" for the leak, thus causing the Pirani reading to fall. This is then followed by a sharp rise in the Pirani reading, as the Acetone is sucked into the vacuum enclosure through the leaking site. such behaviour is positive identification of a leaking site; The leak must then be cured, either by tightening the connection, or by replacing the seal at this connection.

- Check for vent valve leakage.
- Check that the foreline trap is not saturated and requires new molecular sieve.
- Check the operation of the Rotary pump.

On the Penning gauge

The Penning gauge will not reach its normal operating pressure if

- There is a leak on the mass spectrometer vacuum enclosure.
- There is a high content of atmospheric gases drawn into the mass spectrometer via any of the inlet capillaries.
- Helium is not flowing out of either the reference or the sample open split (Check the Helium supply bottle)
- The Turbomolecular pump is in Stand-by.
- The Turbomolecular pump is faulty and not pumping efficiently.
- The Penning gauge or its supply control unit is faulty. Note: If the vacuum reading is too low then the Penning gauge probably needs cleaning.

I cannot achieve the recommended background levels

For Argon

A normal background for Argon should be better than $1\text{E-}11$ A. If this is not achieved, then this is indicative of a leak from atmosphere. The leak (leaks) must be located. The most efficient way to locate such a leak is to use Argon. Please refer to the subsection titled Cleanliness of the vacuum and leak checking in section 6, where the procedure is fully described. It must be remembered that ingress of atmospheric gases can occur anywhere as far as the gas supplies bottles, and it is not always sufficient to leak check around the mass spectrometer and the elemental analyser.

The purity of the gases specified (both He and CO_2) is such that the levels of Argon in these gases is insignificant, and should make only a small contribution to the background levels, well below the normal operating levels.

On extremely rare occasions, it has been observed that some of the supply gases may contain Argon well in excess of the suppliers specifications. This is a very, very rare occurrence, but one that cannot be ruled out completely. Should such a situation arise, then the Argon background may well exceed the normal operation level, although no leak is present.

In this most unlikely event it can only be identified by actually swapping the bottle itself.

CO_2 and N_2

The normal operating background levels for CO_2 and N_2 , should not exceed $5\text{E-}11$ A, with the elemental analyser combustion and reduction tubes set to temperature.

If background levels, exceed this value, then it is most likely caused by outgassing of contamination within the combustion and reduction tubes soon after they have been changed. The backgrounds in this case should drop to normal in about 1 hr.

The background scan is dirty

With prolonged use, the ion optics enclosure may become contaminated. This can be detected by performing background scans.

As long as a reasonable mass spectrometer peak shape is retained, and the quality of data is not affected in anyway, then no action should be taken.

However, if the contamination present in the vacuum enclosure, affects the quality of the results, then the Ion source, the flight tube the source and collector housings must be thoroughly cleaned. Please, refer to the Mass Spectrometry repairs section. This section describes how to carry out various repairs to the mass spectrometer.

I cannot operate the valves

The most common reasons are:

- The compressed air supply is at the wrong pressure.
- There is no compressed air reaching the valve heads.
- The valve cable is disconnected.
- The valve cable is connected to the wrong socket. J5 is for Trace gas preconcentrator only. All other prep systems connect to J6.
- The solenoid valve, corresponding to the valve being actuated, is faulty
- The valve drive board inside the system controller is faulty.

I cannot get a good mass spec peak shape

Any of the following conditions would cause a poor mass spectrometer peak shape:

- Contamination in the Ion Source.
- Contamination in the flight tube.
- Poor source tuning parameters.
- Wrong location for the magnet.
- Faulty source controller unit.

I cannot get stability

The reference gas pulses have an abnormal shape

Degradation in the shape of reference pulses has several causes, almost all of these, require that the reference gas injector be re-built. Please refer to this subsection earlier in this section.

The following list indicates how problems may arise within the reference gas injection assembly:

- The GVF 003/004 ferrule, which constitutes the seal for the ON/OFF action of the MOVPT valve, is of a relatively soft material. This valve is used intensively and after prolonged usage, the sealing face of the ferrule becomes damaged. It needs to be replaced.
- The 25 μ m capillary used to inject the CO₂ gas into the reference flow of Helium, is becoming partially blocked with ferrule or glass dust and needs replacing.
- If instability has occurred, immediately after the reference gas injector has been rebuilt, then check that the correct size capillary has been used, that the 0.006" stainless steel capillary, has been mounted back into the assembly, and that all the recommendations described in the Rebuilding the reference gas injector section have been observed.
- Check that there are no leaks at any of the connectors within the entire assembly.

The reference gas pulses have a normal shape

Please make the following checks:

- Have the elemental analyser combustion tubes been changed recently. If so wait one hour after temperatures have been attained before carrying out a sequence of stability tests.
- Using Argon, leak check the entire system.
- Check that the mass spectrometer is tuned correctly, in particular that the HT is centred on the mass spectrometer peak shape.
If the ambient temperature changes significantly, it is possible to drift off peak centre. This is a very common cause for instability.
- If the mass spectrometer has recently been switched off, please, allow at least one hour for all the components, to stabilise.
- If the vacuum enclosure has recently been exposed to atmospheric gases, then it may take up to one hour for stability to be achieved on the reference gas ratios. This is usually indicated by steadily falling ratio values
- Check the long term stability of the ratios by performing a time scan of the ratios. Please refer to section 6. which describes how to monitor the mass spectrometer stability. The long term behaviour of the ratio trace is very indicative of the reason for mass spectrometer instability.
The ratio trace obtained may display any of the following characteristics:
 - Steady noise band, in excess of $\pm 0.1\%$.
The problem is most likely, an electronics fault:
 - Source supplies are unstable
 - HT supply is unstable
 - Head Amplifier is unstable

It is advisable to contact the customer service department at Micromass

- Random glitches on an otherwise steady noise band indicate, a source filament problem.
- The ratio trace has a slow sinusoidal shape. This is normally indicative of a pumping problem.
- Air conditioning affecting net gas capillary.

I cannot get linearity

- A trivial cause

An apparent 'non linear' effect is sometimes reported.

If the reference gas, which is being used to test the linearity of the system is contaminated by a constant source of contaminant, then depending on the pressure of the reference gas the contamination will be more or less significant. We are therefore concerned with contamination occurring beyond the reference gas pressure regulator.

Apart from leaks of atmospheric gases at the various connectors, the most common source of contamination is a leak between the pneumatic head and the body of the reference gas valve.

A leak at this site can occur in one of two ways:

- The seal between these two compartments is leaking. See the subsection Rebuilding the reference gas injector earlier in this section.

IsoPrime-EA User Manual

- The compressed air pressure exceeds the recommended value.

Important note: Please check that the non linearity effect observed is not caused by the phenomenon described above.

If the cause of the non linearity is not certain, the customer service department at Micromass UK Ltd MUST be contacted.

I cannot get precision on the EA

Ensure that the system is both stable and linear, using the stability and linearity tests described in section 6.

- The elemental analyser combustion/reduction tubes may need regenerating or replacement. The elemental analyser water trap may need regenerating. The combustion tube may contain too much ash.
- The tin sample capsules may contain variable amounts of organic contaminants. This shows as variable CO₂ yield on the blank samples.
- The method file timings are not optimum.
- Check for leaks within the EA and all interfacing connections

I cannot get accuracy

Ensure that the system is both stable and linear and that there is precision of measurement on a standard material

The inaccuracy is constant across a variety of standards

- Check the reference gas calibration.
- Check that there are no leaks within the reference gas assembly.
- Check that there is a continuous flow of CO₂ gas through the reference gas purge. If no flow is recorded, then, please purge the CO₂ line with a high flow initially, then reducing this flow to 5 ml min⁻¹ as described in section 6.
- Check for possible contamination in the EA and all interfacing lines.

The inaccuracy is random across a range of standards.

- The combustion tube needs regenerating or the ash removed.
- The chemical water trap needs regenerating
- Check the cleanliness of the EA and all interfacing capillaries and connectors.
- Check for possible leaks within the EA

I have poor sensitivity

Sample sensitivity is poor, reference gas sensitivity is OK

The mass spectrometer is not responsible for the loss of sensitivity in this case. The problem resides with the EA or its interface.

- Firstly, check for leaks, at all the connections within the EA.
- Loss of chromatography related to dead volumes.

Both sample and reference gas sensitivities are poor

The problem resides with the mass spectrometer. There are very many possible reasons for this and only the most common causes can be described here. If the problem is not rectified, please, contact the customer service department at Micromass UK Ltd stating clearly all the checks that have been made

- Check the source tuning parameters
- Check the mass spectrometer peak shape.
- Check that the Source Electronics respond correctly to the software inputs i.e. Alter the value of each parameter in turn from the data system and check that the values shown on the analogue dials on the front of both the source controller and the source electronics, correspond to the values that have been selected in the tune source parameters window.
- Other causes arise from poor source filament position, deterioration of the source filament, and inefficient pumping.

Sample sensitivity good, Reference sensitivity poor

- Blockage in the reference line.
- Contaminated reference gas
- Too much interface helium flowing in the reference gas box.

I have no sample peak

No sample peak on MS and no signal when RG is open

The problem here is obviously with the mass spectrometer. There are multiple reasons:

- The isolation valve is closed
- The source is not switched ON.
- The mass spectrometer is not tuned to CO₂.
- The wrong tuning file is loaded.
- The magnet supply is not turned ON or is faulty.

If after checking these points there is still difficulty, please, contact the customer service department at Micromass UK Ltd.

No sample peaks on MS, Ms OK, no sample peak on TCD.

The problem here resides in the elemental analyser.

The most common causes are:

- The carousel is not dropping the samples
- The start instruction is not functioning.
- The start stop signal lead has become detached.
- The TCD filament is not switched on.
- The elemental analyser is not in the 'ready' state.

No sample peak at MS, MS OK, sample peak at TCD OK

The problem here is almost certainly the splitter valve being closed.

Sample peak at MS OK, no sample peak on the TCD

The problem here is almost certainly a very large negative offset on the TCD or that the TCD signal lead to the mass spec has become detached.

I have poor chromatography

On both the TCD and the mass spectrometer

This is a pure elemental analyser problem. The most common reasons are

- Poor packing of the reaction tubes
- Poor quality of reaction catalysts particularly the reduction copper.
- GC column temperature too high
- The flow rate through the elemental analyser too high.
- The purge on the carousel is switched off.
- The GC column is contaminated. (Rare)

On the mass spectrometer alone

If there is good chromatographic quality, when inspecting the trace on the TCD, and poor quality on the mass spectrometer, then a component beyond the effluent outlet of the elemental analyser is causing the problem. If the nitrogen peak is good but the CO₂ is bad then suspect a contamination somewhere in the effluent line to the mass spec.

System Controller Error Messages

The errors tabulated below are reported by the system controller as numeric error code, and translated into the text description by the data system software. In the event of an error, the description, along with the command which caused the error, is displayed in the Message Window. If the error code is not recognised by the software, it will be displayed without a text description.

Possible causes are shown below for each type of error. 'Software Errors' are those generated by a bug in the software suite, and should be reported to the Customer Care department.

When reporting an error message to the Customer Care Department, always note down the whole line which appears in the message window, not just the error message itself. It is helpful, if you also note down:

- The operation that was being performed (if any).
- Any suspect behaviour of the software (e.g. Erroneous monitor window data)
- Any message preceding or following the error.

| Code | Description | Possible cause |
|-------------|----------------------------------|---|
| !0300 | invalid command | Software errors Incompatibility between software and firmware versions |
| !0301 | invalid mnemonic | Incorrect mnemonic used in a sequence Old version of firmware, which does not support new prep. system Mimic diagram does not match actual hardware configuration |
| !0302 | invalid argument | Incorrect mnemonic used in a sequence Parameter in sequence or parameter file out of range |
| !0333 | invalid number of parameters | Software error |
| !0400 | invalid beam mask | Software error |
| !0401 | invalid integration time | Integration time in scan window out of range |
| !0405 | bad acquisition command sequence | Software errors |
| !0411 | invalid scan step | Step parameter in scan window out of range |
| !0600 | hardware not present | Incorrect electronics configuration |
| !0601 | invalid hardware type | Errors in configurable mnemonics table |
| !0602 | invalid hardware channel | Errors in configurable mnemonics table |
| !0603 | invalid operation | Software errors |
| !0604 | channel already in use | Software errors |
| !0610 | internal limit reached | Firmware has run out of data space to perform a specific operation |
| !0611 | no more mnemonic space | Configurable mnemonics table too big |
| !0620 | no acquisition hardware | Missing accessory |

Electronics

The following problems may be caused by faults in the electronics systems:

System Crashes

As with any high voltage system, occasional flash-overs (caused by dirty source, high analyser pressure, etc.) can occur. In some circumstances this may cause the microprocessor in the system controller to 'crash'.

Signs that the system controller may have crashed are:

The software error message 'System Controller Not Responding' appears on the PC.

Select OK. If the 'System Controller Not Responding' error appears again it will be necessary to re-boot the system controller by unplugging and reconnecting the mains lead to the unit.

Restart the software.

Problems Controlling the Source/HT Units

If the source cannot be switched on from the software, the most likely cause is a system controller crash as described before. Other possible causes are:

- Turbomolecular pump is not running at full speed.
- The analyser pressure is too high.
- Source connector J1 is not in the correct socket or is not connected.

Magnet Supply Problems (where applicable)

If attempting to change the magnet current results in an 'Invalid Mnemonic' error message or is 'greyed' out in the tune source dialog, the system controller is incorrectly configured.

If the magnet is off despite the programmed current, the possible causes are:

- Cables incorrectly fitted.
- Incorrect voltage setting on power supply
- Blown fuse in power supply.
- Magnet Supply failure.

If the magnet current appears to be full on despite the programmed current, disconnect the power to the magnet controller at its mains inlet to avoid damage to the electromagnet. Check that the cable between the system controller and the magnet supply is intact.

Beam Problems

If it appears impossible to get any beam readings in the monitor window, check that the Head amp. power supply and fibre optic connections are intact.

The head-amp power supply status can be queried using the firmware *read* command using a terminal emulator. See below for details.

Fault Diagnosis Using a Terminal Emulator

If a fault has occurred which could be caused by either software or system electronics, communicating with the electronics directly can help. OS/2 has a terminal emulator which can be used for this. This will usually have been

IsoPrime-EA User Manual

configured during factory testing, however complete instructions are included in the appendix.

- Shut down the data-system software.
 - Open the **OS/2 System** window.
 - Open the **Productivity** window.
 - Start-up the application **PM Terminal**
 - Select the session configured for system controller communications.
- Any characters typed are sent directly to the system controller. A full list of commands is included in the Firmware Manual (included in the appendix), but here are some tips:
- Press the space bar before typing each command to get an echoing prompt.
 - use the read command '**r**' and write command '**w**' to access hardware which is suspect (valves etc.). A full list of mnemonics is included in the firmware manual.
 - The internal commands '**i...**' can be used to get detailed system information. If the Isotopic Analysis factory is contacted with a problem these commands may need to be used.
 - Always leave the cursor at the left-hand margin - not at the prompt - before leaving PMTerminal.

General Notes on Fault Finding

Here are a few very brief guidelines to help you when fault finding.

- **ALWAYS** check every possibility outside the vacuum before opening up the system.
- **ALWAYS** allow plenty of time for the vacuum to come down before assuming that there is a leak.
- **ALLOW** the head amplifier at least an hour to warm up before taking measurements.
- **ALLOW** the magnet at least an hour to warm up before taking measurements.
- **ALWAYS** double check especially when swapping components.
- **ALWAYS** bear in mind that poor results with the VG-ISOCHROM-EA. are only very rarely caused by mass spectrometer or electronics problems. The fault almost invariably, resides within the EA. By far the major causes for poor results, are contamination, leaks, poor combustion, poor chromatography. All of these can be rectified through the maintenance procedures described in this manual.

When the source of the problem has been located the repair must be carried out meticulously to ensure the cure is successful.

Contact details

If, after following the guidance contained within this chapter, you are unable to rectify the fault on your instrument; please contact the Customer Service Department at:

Micromass UK Ltd.
Floats road,
Wythenshawe,
Manchester,
M239LZ,
UK

Phone ++44 (0) 161 718 4575

Fax ++44 (0) 161 718 4660

Or contact your local Micromass representative.

Practical considerations

The aim of this section is to introduce the user to a number of practical aspects associated with sample running. Weighing samples, characterising samples, minimising memory effects between samples are typical aspects which can affect the quality of data produced. It must be heavily emphasised however that the content of this section represents just a small introductory contribution to a much wider sample management discussion effort. The ideas discussed are not intended to be absolute articles of faith, rather they should be used to generate discussion on these and many other subtle aspects of sample analysis which are bound to occur from time to time.

Using the micro balance.

Proper use of a quality micro balance is absolutely essential in order to obtain good reproducible data. A good quality balance is one which can measure to 1 or 2 μg . 1 μg is better but there can be a huge price differential between the two. In either case good performance can only be guaranteed if the balance is placed on a proper solid balance table insulated from vibrational sources. Due to the sensitivity of the Isochrom EA it often happens that sample sizes of around 0.1mg are weighed out. In this case an inherent accuracy of 2 μg can be quite significant to the system when calculating total N or C. For this reason, reference material, should, where possible, be matched with the sample in terms of %N or %C. For example, it makes no sense to attempt to quantitatively measure 1% C using a reference material containing 70% C. Many nitrogen samples, particularly in soils and sediments may contain only 0.1% N so a suitable homogeneously ground lab soil standard containing < 1% N should be used.

IsoPrime-EA User Manual

Balance Equipment

Heavy 6" pointed tweezers

Spatula

EA tin capsule holder

Variety of different size tin capsules

Plastic sample tray 96 sample capacity

Large supply of tissue paper

Procedure

Clean the points of the tweezers

Lift out the sample balance tray and wipe clean and replace

Wipe the spatula clean

Place fresh sheet of tissue on the bench

Place spatula on the paper

Clean the capsule holder and place on the paper

Place a new tin capsule on the balance and tare the weight

Load the capsule with the sample material

Weigh the sample. If sample weight not correct then fiddle about until it is.

Place the loaded capsule on the surface of the sample holder

Using the tweezers close the top of the capsule tightly and fold over.

Squeeze down tightly on fold 1 then fold again

Compress the sample till a compact solid ball is produced

Place in the sample tray.

Repeat process for 3 replicates.

For a new sample repeat the cleaning steps.

Note:

At first sight it may seem reasonable to suppose that smaller tin capsules will contain less carbon blank and in fact this is generally the case. As such common sense says to use the small cups when measuring small samples. However the variation in the blank is inherent to both carbon content and associated delta value of this content so the important thing is to have consistency in both.

Managing memory

Inter sample memory is a problem which is associated entirely with the combustion process of the elemental analyser. The elimination of memory is both difficult and unpredictable. Sometimes the combustion conditions in the oxidation tube are perfect resulting in negligible memory, at other times the memory is pronounced. The following is one explanation as to why this is so.

Consider the surface of the combustion zone, i.e., where the sample lands after the carousel has dropped it into the furnace. If the material making up the surface has poor thermal insulation properties then it is reasonable to assume that as the flash combustion takes place, heat will be conducted away at the contact point between the sample and the surface. It follows that the contact point never reaches the flash combustion temperature of 1700°C and so a small but significant portion of the sample may not combust and is left as a residual to be mixed with the next sample. If however the surface is a very good thermal insulator then heat will not be conducted away resulting in all of the sample, flash combusting at 1700°C. The next question is "what sort of materials are involved"? If the oxidation tube is packed according to the EA manual it will have a top surface consisting of quartz wool. Leave the quartz wool out and it will be a chromium oxide surface. Samples containing little non combustible material will gradually build up a surface associated with the tin capsules. Samples such as soil or sediments on the other hand build up ash deposits. All of these surfaces will have different thermal conductivity characteristics resulting in possibly different memory characteristics. Regardless of the memory characteristics prevailing at the time, it makes sense to construct the autorun in such a manner that any memory effects are minimised.

For example

The samples have been characterised and found to have the following approximate isotopic values

| | |
|---|----------------|
| A | 0.3654 At% N15 |
| B | 1.2345 At% N15 |
| C | 0.8756 At% N15 |
| D | 0.4659 At% N15 |
| E | 1.7439 At% N15 |

It would make sense to construct the batch run in such a way that the jump in enrichment between sample types is reduced to a minimum. As such, the batch file may look like the following.

| Sample | Replicates |
|--------------|------------|
| Blank | 3 |
| Elem Ref n/a | 3 |
| A | 3 |
| D | 3 |
| C | 3 |
| B | 3 |
| E | 3 |
| Elem ref n/a | 3 |

Note that the blank values are measured at the beginning of the batch while the residual memory is at natural abundance level. This is followed by

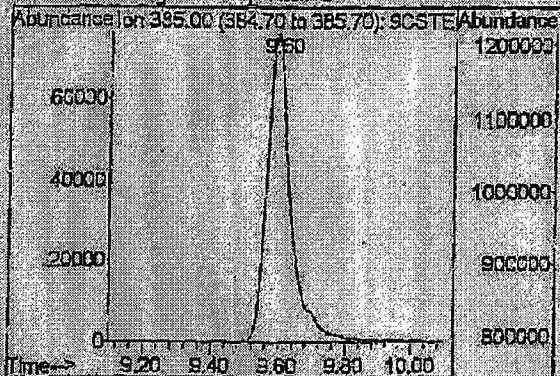
IsoPrime-EA User Manual

samples increasing in enrichment as the run progresses. Never insert dummy or wash samples in the middle of a run like this as it will serve only to increase the step in enrichment to the next sample. The only time wash samples should be used is to come back down to natural abundance level following the analysis of enriched samples. The elem ref samples at the end of the above run will be valid for quantitative measurements though perhaps not much use as isotopic standards. In the same way, introduction of blanks at the end of this batch will not result in a valid isotopic correction.

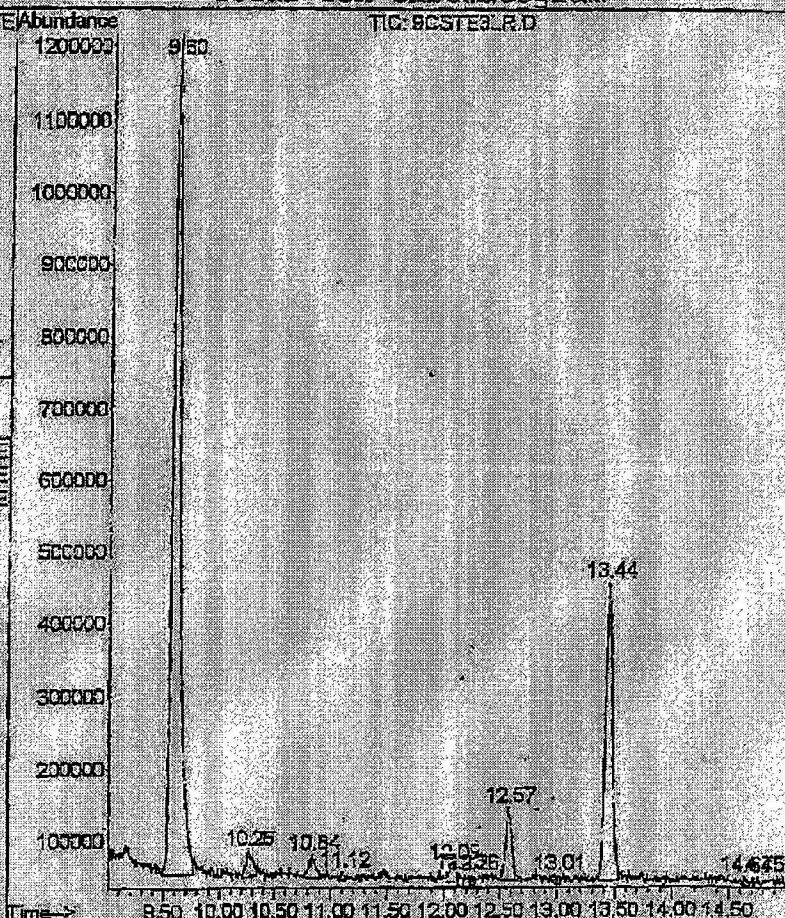
Data File : K:\CHEM\AAS\CONFIRM\MSDA13\9CSTE3LR.D
Acquired : 29 Jun 8:33 pm
Instrument : MSDA13
Misc Info :
Sample Name: TE 4:1 STANDARD LIN

Method File: ANAB97LS
Vial Number: 86

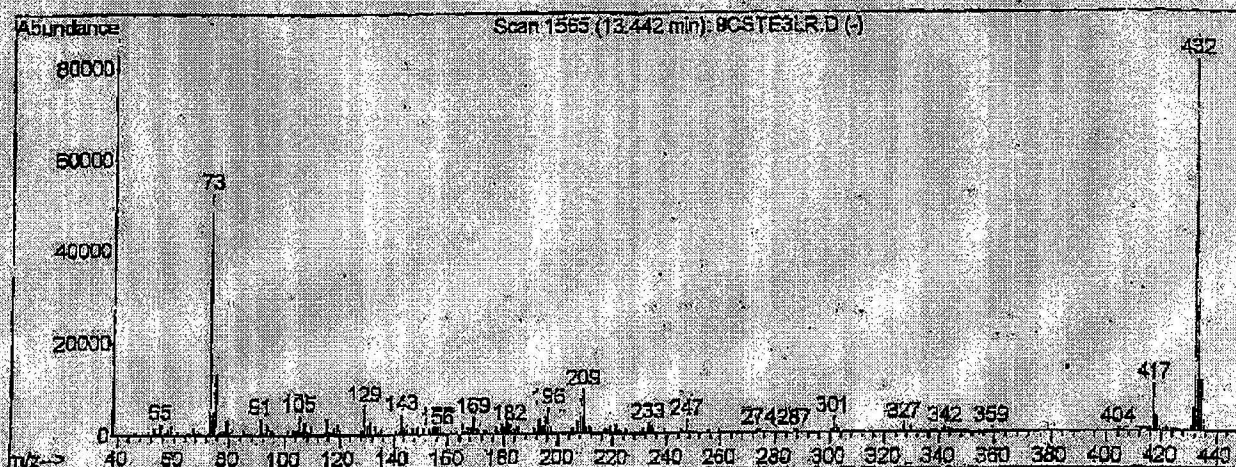
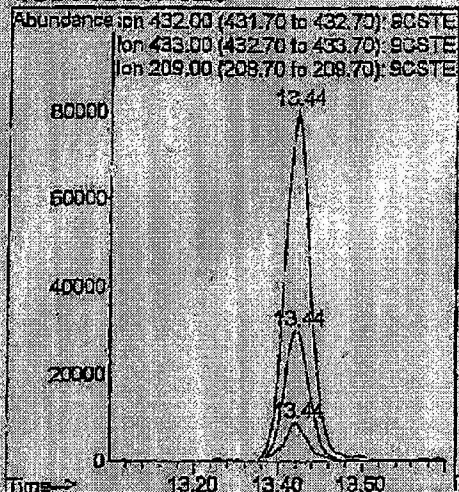
ISTD Ethylmorphine



Total Ion Chromatogram



Testosterone



Mul301 *6/30/01*

INDEX OF TRIAL EXHIBITS
LANDIS/2006 TOUR DE FRANCE
AAA No. 30 1090 847 06
T 56563-00001

| Bates Range | Description of Exhibit |
|--------------------|---|
| GDC0001 – GDC0003 | July 27, 2006 letter from USADA to Floyd Landis re Urine Sample |
| GDC0004 – GDC0005 | July 31, 2006 letter from Floyd Landis to USA Cycling |
| GDC0006 | May 8, 2006 Press Release from UCI re Adverse Analytical Finding |
| GDC0007 – GDC0022 | Submission to USADA Independent Anti-Doping Review Board from Howard Jacobs |
| GDC0023 | September 15, 2006 letter from USADA Review Panel to Terrence Madden |
| GDC0024 – GDC0029 | GC/C/IRMS and GC/MS in "Natural" Steroids Testing |
| GDC0030 – GDC0031 | Chanie de possession |
| GDC0032 – GDC0033 | Chain of Custody Documentation |
| GDC0034 – GDC0053 | The Endocrine System in Sports and Exercise |
| GDC0054 – GDC0055 | Doping Expert thinks Landis result 'doesn't add up' |
| GDC0056 – GDC0061 | Written Statement of Gary Wadler for Congressional Hearings on Major League Baseball & the use of Performance Enhancing Drugs |

| Bates Range | Description of Exhibit |
|--------------------|--|
| GDC0062 – GDC0063 | WADA Panel Member Wadler Q&A |
| GDC0064 – GDC0133 | Anti-Doping Examination Regulations |
| GDC0134 – GDC0160 | Award rendered by The Court of Arbitration for Sport in arbitration between USADA and Tim Montgomery |
| GDC00161 – GDC0192 | Sentence Arbitrale rendue par le Tribunal Arbitral Du Sport |
| GDC0193 – GDC0218 | Arbitral Award delivered by the Court of Arbitration for Sport in arbitration between UCI v. Inigo Landaluce Intxaurreaga and RFEC |
| GDC0219 – GDC0232 | International Federation of Clinical Chemistry, Testing urine for drugs |
| GDC0233 | WADA Technical Document – TD2003LCOC |
| GDC0234 | Abuse of androgens and detection of illegal use |
| GDC0235 – GDC0246 | Anti-Doping Convention (T-DO) Meeting of the Advisory Group on Science |
| GDC0247 – GDC0248 | GCMS: How Does It Work? |
| GDC0249 – GDC0271 | Anabolic Steroids |
| GDC0272 – GDC0278 | The Effects of Supraphysiologic Doses of Testosterone on Muscle Size and Strength in Normal Men |
| GDC0279 – GDC0288 | Testosterone dose-response relationships in healthy young men |
| GDC0289 – GDC0323 | General requirements for the competence of testing and calibration laboratories |
| GDC0324 | April 26, 2007 e-mail from Michael Henson to Maurice Suh re Sport 24 – French |

| Bates Range | Description of Exhibit |
|--------------------|---|
| GDC0325 | April 26, 2007 e-mail from Michael Henson to Maurice Suh re Sport 24 – Translated |
| GDC0326 – GDC0327 | April 26, 2007 e-mail from Michael Henson to Maurice Suh re L'Equipe 4/24 – French |
| GDC0328 | April 26, 2007 e-mail from Michael Henson to Maurice Suh re L'Equipe 4/24 – Translated |
| GDC0329 – GDC0330 | April 26, 2007 e-mail from Michael Henson to Maurice Suh re L'Equipe 11/14/06 – French |
| GDC0331 – GDC0332 | April 26, 2007 e-mail from Michael Henson to Maurice Suh re L'Equipe 7/30/06 – French |
| GDC0333 – GDC0373 | The World Anti-Doping Code, International Standard for Testing, version 3.0 |
| GDC0374 – GDC0395 | World Anti-Doping Code, Guidelines for Urine Sample Collection, version 4.0 |
| GDC0396 – GDC0400 | WADA Technical Document – TD2003IDCR |
| GDC0401 | UCLA Olympic Analytical Laboratory Testosterone Confirmation Report |
| GDC0402 | GC/IRMS – Results |
| GDC0403 | Documentation Analysis Report 202/03 |
| GDC0404 – GDC0424 | The List, The 2005 Prohibited List, International Standard |
| GDC0425 – GDC0430 | Document of unknown source or topic |
| GDC0431 – GDC0450 | Recent Advances in Doping Analysis |
| GDC0451 – GDC0452 | June 21, 2001 excerpts from letter to Clients of the UCLA Olympic Laboratory re Carbon Isotope Ratio measurements, Update 3 |
| GDC0453 – GDC0496 | World Anti-Doping Code |

| Bates Range | Description of Exhibit |
|---------------------|---|
| GDC0497 – GDC0509 | The role of measurement uncertainty in doping analysis |
| GDC0510 – GDC0513 | Appendix D: Error List |
| GDC0514 – GDC0521 | February 13, 2007 <i>Declaration of Simon Davis</i> |
| GDC0522 | IsoPrime EA User Manual |
| GDC00523 – GDC00531 | Testosterone Confirmation Report |
| GDC00532 – GDC00533 | Documentation Analysis Report 202/03 |
| GDC00534 | Carbon Isotope Ratio Report |
| GDC00535 | Drug Testing Report USADA |
| GDC00536 – GDC00537 | June 21, 2001 letter to Clients of UCLA Olympic Laboratory |
| GDC00538 – GDC00596 | Agilent Gas Chromatographs – Fundamentals of Gas Chromatography |
| GDC00597 – GDC00618 | Commonly Practiced Quality Control and Quality Assurance Procedures for Gas Chromatography / Mass Spectrometry Analysis in Forensic Urine Drug-Testing Laboratories |
| GDC00619 | Hormonal Control of Testicular Function |
| GDC00620 | Table showing Testosterone Enanthate (100 mg IM weekly) |
| GDC00621 –GDC00632 | Effect of multiple oral doses of androgenic anabolic steroids on endurance performance and serum indices of physical stress in healthy male subjects |
| GDC00633 – GDC00642 | Exogenous testosterone, aggression, and mood in eugonadal and hypogonadal men |

| Bates Range | Description of Exhibit |
|---------------------|--|
| GDC00643 – GDC00651 | AA2500 Testosterone Gel Normalizes Androgen Levels in Aging Males with Improvements in Body Composition and Sexual Function |
| GDC00652 – GDC00656 | Detection of anabolic steroid administration: ratio of urinary testosterone to epitestosterone vs the ratio of urinary testosterone to luteinizing hormone |
| GDC00657- GDC00663 | A Single Dose of the Potent Gonadotropin-Releasing Hormone Antagonist Acyline Suppresses Gonadotropins and Testosterone for 2 Weeks in Healthy Young Men |
| GDC00664 – GDC00670 | Testosterone administration to older men improves muscle function: molecular and physiological mechanisms |
| GDC00671 – GDC00678 | Oral Testosterone in Oil Plus Dutasteride in Men: A Pharmacokinetic Study |
| GDC00679 – GDC00686 | Exogenous Testosterone or Testosterone with Finasteride Increases Bone Mineral Density in Older Men with Low Serum Testosterone |
| GDC00687 – GDC00733 | World Anti-Doping Agency Independent Observer Report Tour De France 2003 |
| GDC00734 | Unidentified picture |
| GDC00735 – GDC00737 | Floyd Landis Power Data |
| GDC00738 – GDC00765 | Curriculum Vitae Bruce A. Goldberger, Ph.D, DABFT |
| GDC00766 – GDC00769 | Curriculum Vitae Allen C. Lim, PhD |
| GDC00770 – GDC00774 | Dr. Wolfram Meier-Augenstein, CChem, MRSC |
| GDC00775 – GDC00791 | John Kenneth Armory MD, MPH, FACP |
| GDC00792 – GDC00870 | Articles from <i>Bicycling</i> |

| Bates Range | Description of Exhibit |
|---------------------|---|
| GDC00871 – GDC00905 | Computer screen captures |
| GDC00906 – GDC01018 | Computer screen captures; lab results |
| GDC01019 – GDC01055 | Lab results |
| GDC01056 – GDC01079 | Lab logs/results |
| GDC01080 – GDC01907 | Le Tour 2006: Daily Analysis Reports |
| GDC01098 – GDC01106 | Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstenediols |
| GDC01107 – GDC01127 | Applied gas chromatography coupled to isotope ratio mass spectrometry |
| GDC01128 – GDC01133 | Routine Analysis by High Precision Gas Chromatography/Mass Selective Detector/Isotope Ratio Mass Spectrometry to 0.1 Parts Per Mil |
| GDC01134 – GDC01139 | Use of gas chromatography-combustion-isotope ratio mass spectrometry in nutrition and metabolic research |
| GDC01140 – GDC01153 | Department of Communications, Information Technology and the Arts Anti-Doping Research Program Progress Report March 2004 |
| GDC01154 – GDC01163 | Analysis of quantization error in high-precision continuous-flow isotope ratio mass spectrometry |
| GDC01164 – GDC01179 | High-Precision Measurement of ¹³ C/ ¹² C Ratios by On-Line Combustion of GC Eluates and Isotope Ratio Mass Spectrometry |
| GDC01180 – GDC01207 | Some instrumental effects in the determination of stable carbon isotope ratio by gas chromatography-isotope ratio mass spectrometry |

| Bates Range | Description of Exhibit |
|---------------------|--|
| GDC01208 – GDC01239 | High-Precision Continuous-Flow Isotope Ratio Mass Spectrometry |
| GDC01240 – GDC01249 | Analysis of quantization error in high-precision continuous-flow isotope ratio mass spectrometry |
| GDC01250 – GDC01266 | Analytical improvements in irm-GC/MS analyses: Advanced techniques in tube furnace design and sample preparation |
| GDC01267 – GDC01296 | Isotopic Fractionation of Organic Compounds in Chromatography |
| GDC01297 – GDC01304 | Curve Fitting for Restoration of Accuracy for Overlapping Peaks in Gas Chromatography/Combustion Isotope Ratio Mass Spectrometry |
| GDC01305 – GDC01309 | On-Line Recording of $^{13}\text{C}/^{12}\text{C}$ Ratios and Mass Spectra in one Gas Chromatographic Analysis |
| GDC01310 – GDC01312 | Bridging the Information Gap Between Isotope Ratio Mass Spectrometry and Conventional Mass Spectrometry |
| GDC01313 – GDC01318 | High-precision gas chromatography-combustion isotope ratio mass spectrometry at low signal levels |
| GDC01319 – GDC01323 | Derivatization of Organic Compounds Prior to Gas Chromatographic-Combustion-Isotope Ratio Mass Spectrometric Analysis: Identification of Isotope Fractionation Processes |
| GDC01324 – GDC01333 | Journal of Chromatography – Influence of gas chromatographic parameters on measurement of $^{13}\text{C}/^{12}\text{C}$ isotope ratios by gas-liquid chromatography-combustion isotope ratio mass spectrometry |
| GDC01334 – GDC01344 | Isotope-ratio-monitoring gas chromatography-mass spectrometry: methods for isotopic calibration |
| GDC01345—GDC 1346 | L'Equipe Article re Leaked Testing Results |
| GDC01347.01 | Article – "Landis Fails Drug Test After Triumph in Tour de France" |
| GDC01348 | Article – "LeMond: Landis Could be 'Symbol of Change'" |

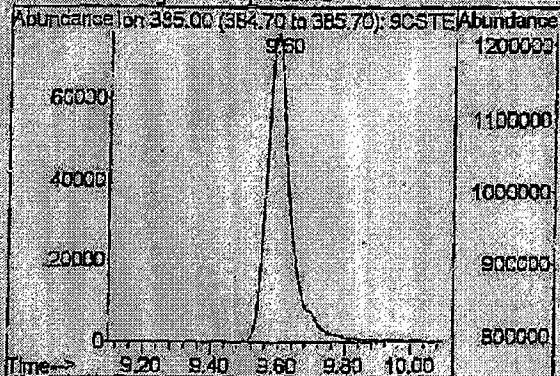
| Bates Range | Description of Exhibit |
|--------------------|--|
| GDC01349.01 | Article – "Landis Fails Backup Test; Tour Title in Jeopardy" |
| GDC01350 | Reprocessing Chart |
| GDC01351 | Montreal Chromatograph |
| GDC01352 | Peter Hemmersbach, Ph.D. Witness Statement |
| GDC01353 | Article – Chicago Tribune |
| GDC01354 | Ayotte Article |
| GDC01035 | UCLA TE Chromatogram |
| GDC01036 | UCLA TE Chromatogram |
| GDC01037 | UCLA TE Chromatogram |
| GDC01038 | Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline |
| GDC01359 | USADA Letter to Joseph Papp dated June 30, 2006 |
| GDC01360 | Article, dated March 14, 2007, "Catlin is leaving UCLA Anti-Doping Lab" |
| GDC01361 | Article, dated March 14, 2007, "Catlin is leaving UCLA Anti-Doping Lab" |
| GDC01362 | UCLA Olympic Analytical Laboratory |

100223775_1.DOC

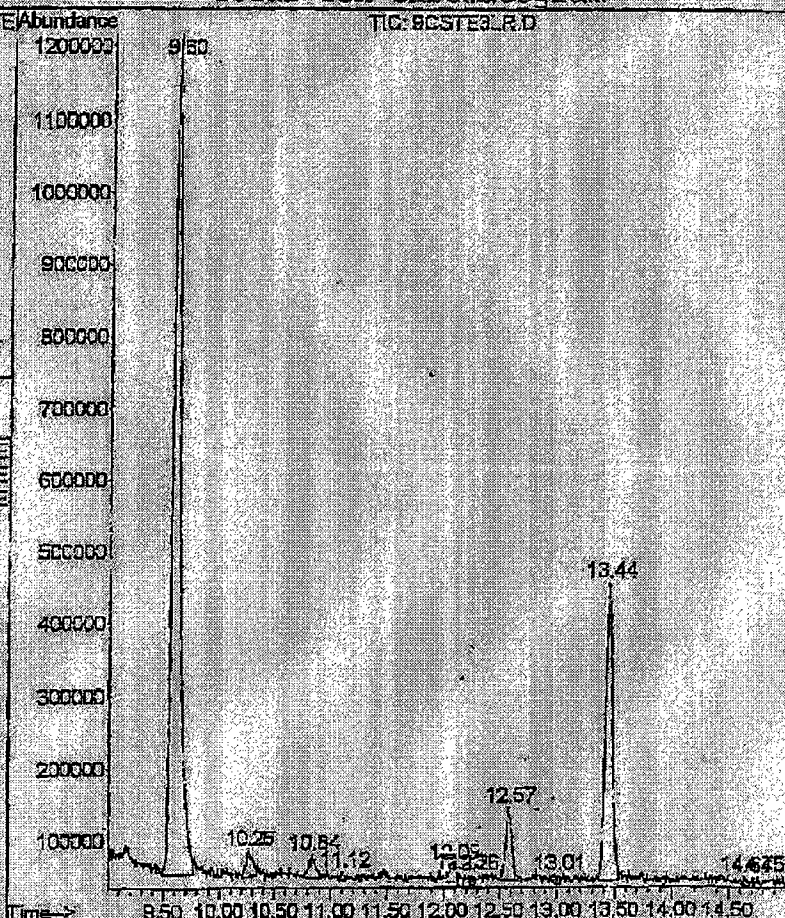
Data File : K:\CHEM\AAS\CONFIRM\MSDA13\9CSTE3LR.D
Acquired : 29 Jun 8:33 pm
Instrument : MSDA13
Misc Info :
Sample Name: TE 4:1 STANDARD LIN

Method File: ANAB97LS
Vial Number: 86

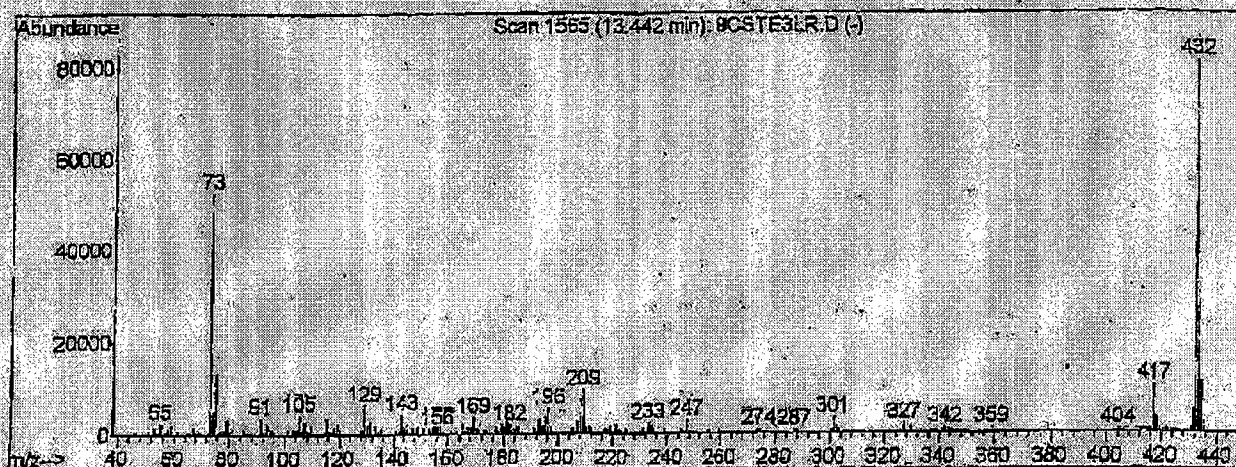
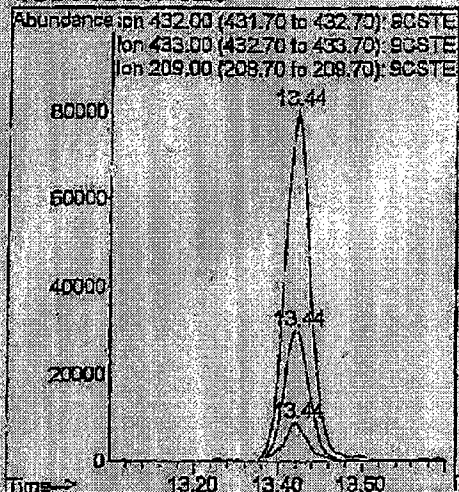
ISTD Ethylmorphine



Total Ion Chromatogram



Testosterone



Mulzol 6/30/00

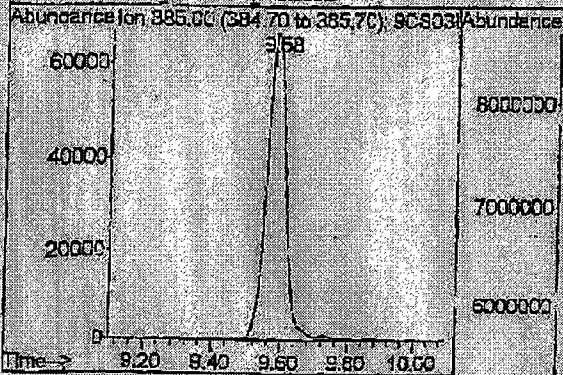
UCLA OLYMPIC ANALYTICAL LABORATORY
Testosterone Confirmation Report

p37

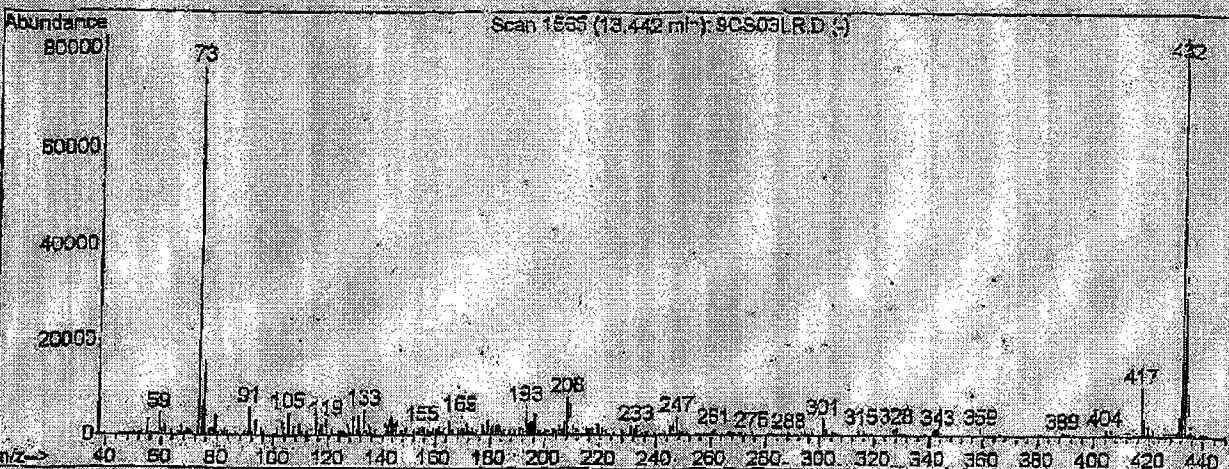
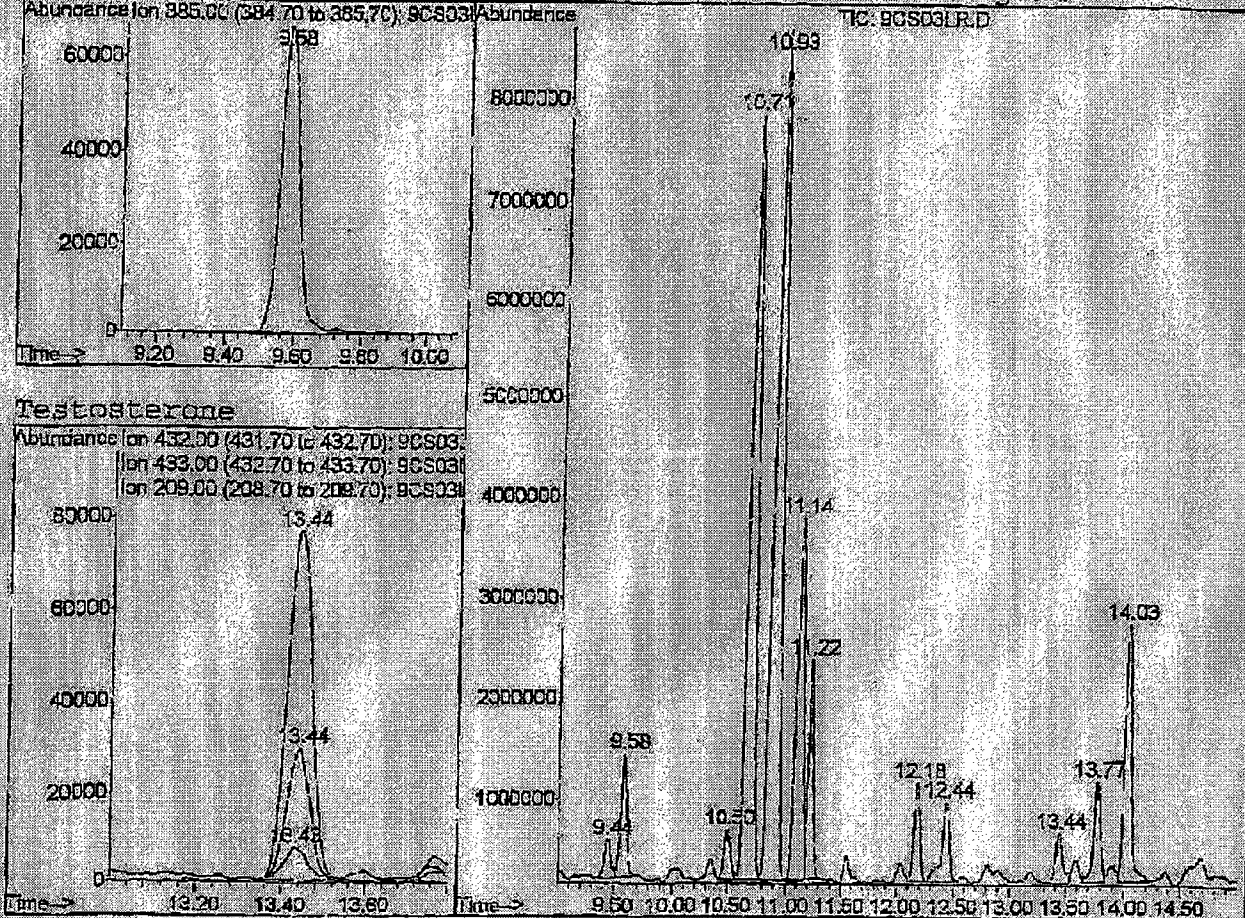
Data File : K:\CHEM\AAS\CONFIRM\MSDA13\9CS03LR.D
Acquired : 29 Jun 6:47 pm
Instrument : MSDA13
Misc Info :
Sample Name: 9CS03 TE A CONFIRMATION LIN

Method File: ANAB97LS
Vial Number: 84

ISTD Ethylmorphine



Total Ion Chromatogram



Handwritten signature and date: 6/30/01

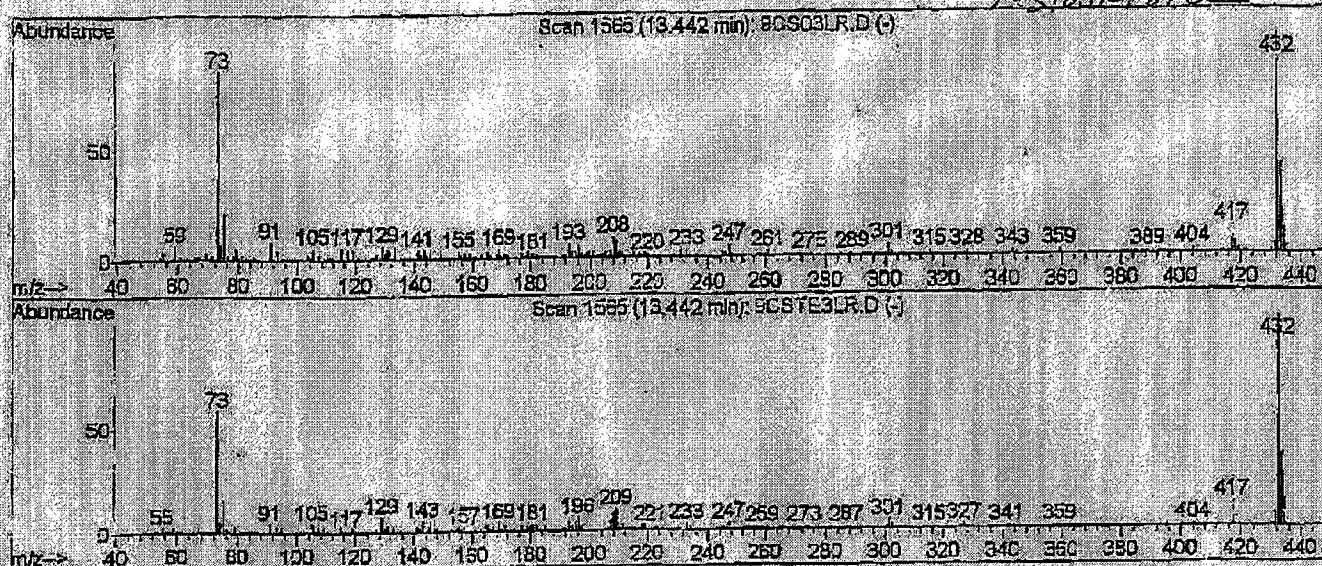
USADA 0039

GDC00524

*** Linear Spectrum Cross Correlation Report ***

>> Cross Correlation for the spectra below = 0.9776 <<

Testosterone



>>> Normalized Tabulation of First Spectrum Printed Above
Result of BIG
Baa

| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 55.05 | 3 | 105.05 | 5 | 169.10 | 4 | 231.10 | 3 |
| 59.05 | 6 | 115.10 | 5 | 171.10 | 2 | 233.20 | 3 |
| 67.10 | 2 | 117.05 | 5 | 176.95 | 2 | 247.20 | 5 |
| 69.10 | 3 | 119.10 | 4 | 179.10 | 3 | 301.10 | 5 |
| 73.10 | 86 | 129.05 | 6 | 193.10 | 6 | 417.30 | 12 |
| 74.10 | 7 | 131.10 | 4 | 194.10 | 3 | 418.20 | 5 |
| 75.10 | 21 | 133.10 | 6 | 195.10 | 3 | 431.35 | 4 |
| 79.00 | 5 | 141.10 | 4 | 196.20 | 5 | 432.25 | 100✓ |
| 81.10 | 2 | 143.10 | 4 | 206.95 | 3 | 433.30 | 41✓ |
| 91.10 | 8 | 155.20 | 3 | 208.10 | 9 | 434.40 | 11 |
| 93.10 | 4 | 165.05 | 3 | 209.10 | 7✓ | 435.30 | 3 |

>>> Normalized Tabulation of Second Spectrum Printed Above
Result of BIG
Baa

| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|--------|--------|--------|--------|--------|--------|--------|--------|
| 55.10 | 2 | 119.00 | 2 | 180.00 | 2 | 209.20 | 10✓ |
| 59.05 | 2 | 129.00 | 7 | 181.00 | 3 | 233.10 | 3 |
| 67.10 | 2 | 130.10 | 2 | 182.10 | 2 | 247.20 | 3 |
| 73.10 | 58 | 131.10 | 3 | 185.10 | 2 | 301.20 | 4 |
| 74.10 | 5 | 133.00 | 2 | 193.20 | 4 | 417.30 | 12 |
| 75.05 | 15 | 143.00 | 5 | 194.10 | 2 | 418.30 | 4 |
| 79.05 | 3 | 165.10 | 3 | 195.10 | 4 | 431.40 | 6 |
| 91.05 | 4 | 169.10 | 4 | 196.20 | 6 | 432.30 | 100✓ |
| 93.10 | 3 | 171.10 | 3 | 197.20 | 2 | 433.30 | 35✓ |
| 105.10 | 4 | 177.10 | 2 | 207.10 | 4 | 434.40 | 13 |
| 115.10 | 3 | 179.10 | 2 | 208.10 | 8 | 435.30 | 3 |

Musol *ms 6/3/80*

UCLA Olympic Analytical Laboratory

'A' CONFIRMATION SUMMARY

Client: USADA
Folder: 7800
UCLA Code: 9CS03

Bottle: [REDACTED]

CONFIRMATION DATA

| Substance: | Testosterone | WADA ISL | | IS = Ethylmorphine | |
|-------------------|--------------|----------|-------|--------------------|---------|
| | Calibrator | Ranges | | NEG QC | 9CS03 |
| GC-MS Datafile | 9CSTE3LR | min | max | 9CSNQCAR | 9CS03LR |
| ISTD RT (min) | 9.60 | 9.50 | 9.70 | 9.60 | 9.58 |
| Compound RT | 13.44 | 13.31 | 13.58 | ND | 13.44 |
| Compound RRT | 1.406 | NA | NA | NA | 1.403 |
| Base peak 432 | 100 | NA | NA | NA | 100 |
| Ion 1 433 | 41 | 33 | 49 | NA | 35 |
| Ion 2 209 | 7 | 2 | 12 | NA | 10 |
| Cross Correlation | NA | NA | NA | NA | 0.9776 |

CONCLUSION:

Sample 9CS03 has a high T/E ratio.

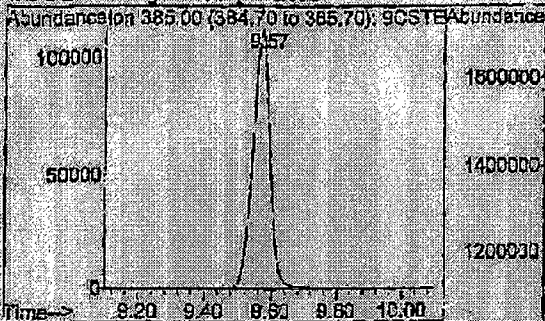
YJ 6/30/ [REDACTED]
no 1/34/ [REDACTED]

USADA 0041

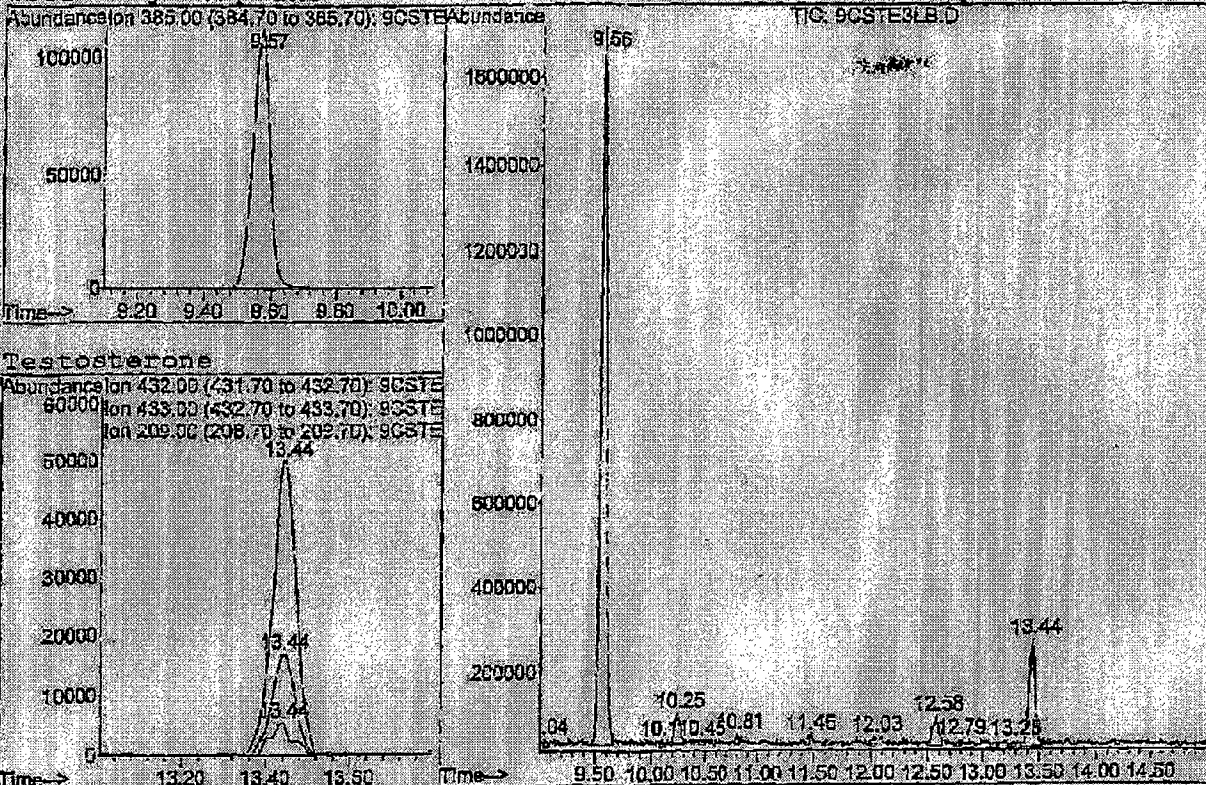
Data File : K:\CHEM\AAS\CONFIRM\MSDA14\9CSTE3LB.D
Acquired : 2 Aug 8:12 pm
Instrument : MSDA14
Misc Info :
Sample Name: TE 4:1 STANDARD LIN

Method File: ANAB97LS
Vial Number: 42

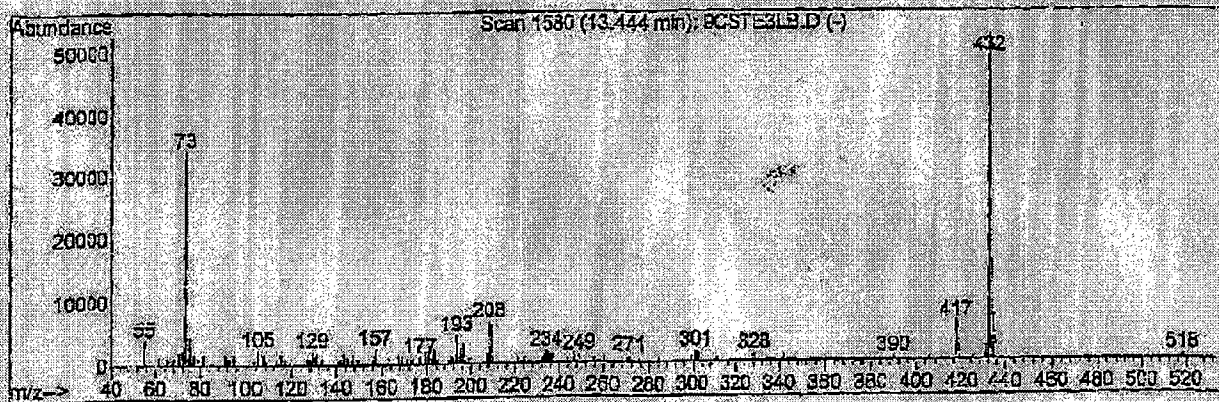
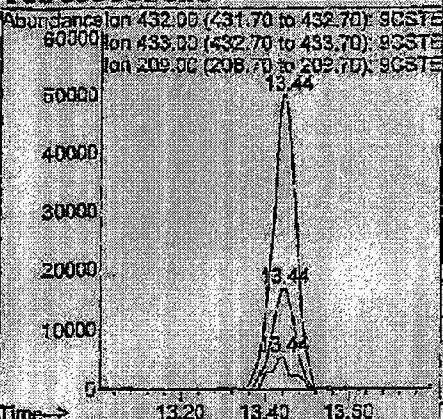
ISTD Ethylmorphine



Total Ion Chromatogram



Testosterone

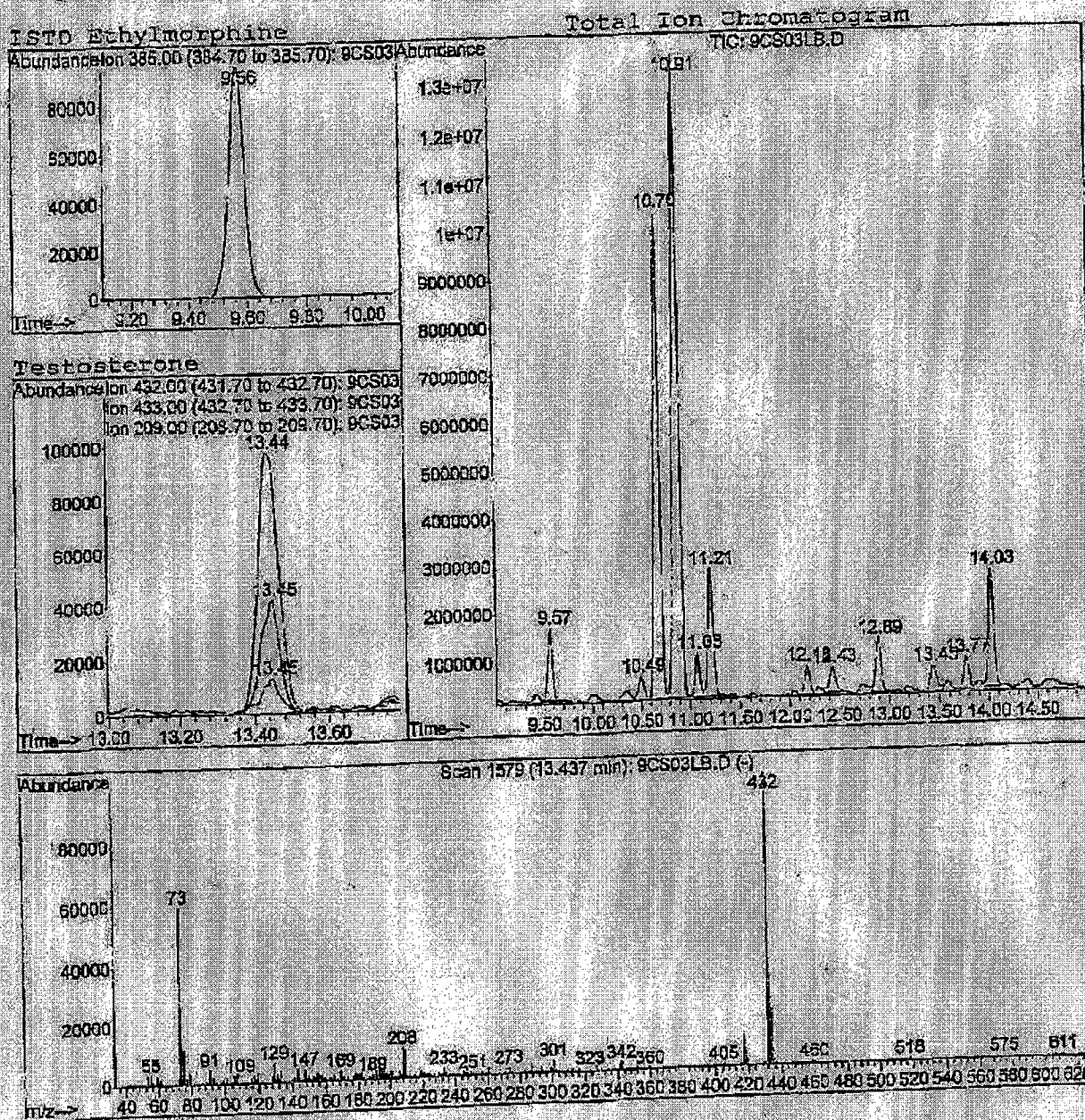


TS 8/31

2/7/10

Date File : K:\CHEM\AAS\CONFIRM\MSDA14\9CS031B.D
Acquired : 2 Aug 7:20 pm
Instrument : MSDA14
Misc Info :
Sample Name: 9CS03 TE 'B' CONFIRMATION L1N

Method File: ANAB97LS
Vial Number: 41



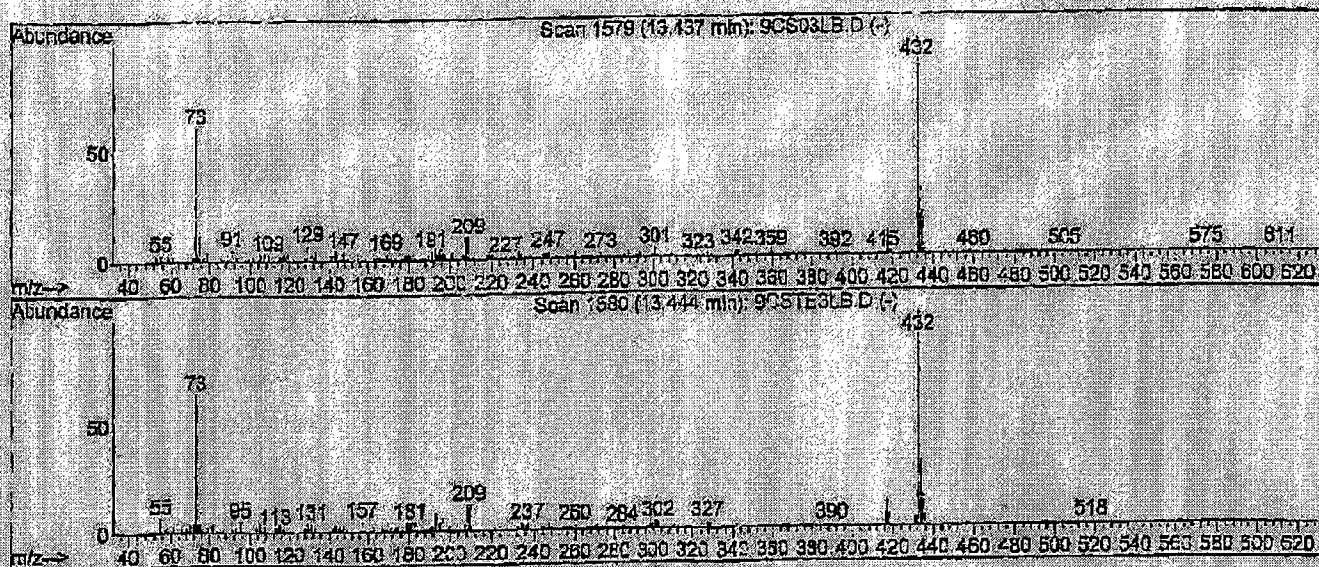
TS 8/31

MS 8/31

*** Linear Spectrum Cross Correlation Report ***

p 64

>> Cross Correlation for the spectra below = 0.9787 <<



>>> Normalized Tabulation of First Spectrum Printed Above
Result of BIG

Baa

| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 53.10 | 3 | 105.10 | 3 | 155.10 | 3 | 209.20 | 10 |
| 55.20 | 3 | 107.90 | 3 | 169.15 | 3 | 233.20 | 3 |
| 59.05 | 3 | 109.10 | 3 | 182.15 | 3 | 247.20 | 3 |
| 61.00 | 2 | 117.05 | 3 | 191.30 | 4 | 287.25 | 3 |
| 71.90 | 2 | 119.20 | 3 | 193.20 | 6 | 301.10 | 4 |
| 73.10 | 61 | 129.05 | 3 | 194.10 | 3 | 342.30 | 3 |
| 74.20 | 4 | 131.10 | 3 | 195.20 | 4 | 417.30 | 11 |
| 75.10 | 12 | 132.05 | 2 | 196.10 | 5 | 418.40 | 5 |
| 77.15 | 2 | 133.05 | 4 | 197.10 | 3 | 432.40 | 100 |
| 79.00 | 4 | 143.10 | 5 | 201.10 | 3 | 433.30 | 31 |
| 91.00 | 5 | 147.30 | 4 | 208.05 | 10 | 434.40 | 20 |

>>> Normalized Tabulation of Second Spectrum Printed Above
Result of BIG

Baa

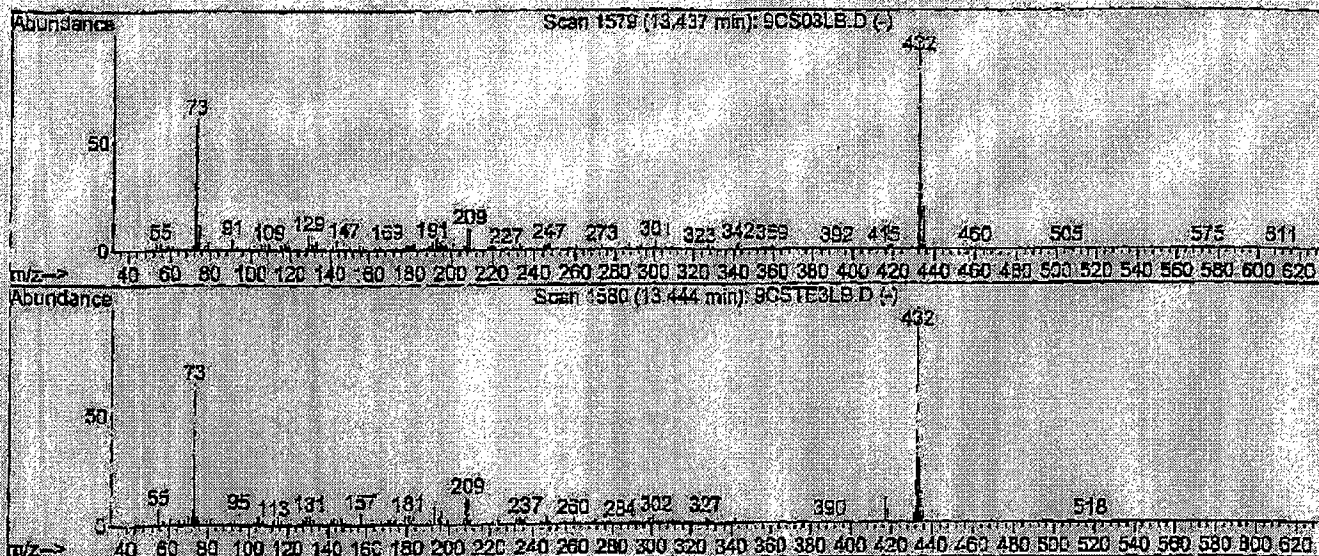
| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 55.10 | 7 | 105.00 | 4 | 160.80 | 4 | 246.80 | 3 |
| 61.20 | 3 | 107.10 | 3 | 183.10 | 4 | 301.10 | 3 |
| 68.00 | 2 | 115.05 | 4 | 193.30 | 8 | 302.50 | 3 |
| 70.20 | 4 | 128.10 | 2 | 195.10 | 4 | 327.30 | 3 |
| 72.00 | 4 | 129.10 | 4 | 196.20 | 5 | 417.20 | 11 |
| 73.10 | 55 | 130.00 | 2 | 199.20 | 2 | 418.30 | 6 |
| 74.10 | 5 | 131.10 | 4 | 207.20 | 3 | 431.20 | 4 |
| 75.10 | 4 | 143.10 | 3 | 208.20 | 12 | 432.40 | 100 |
| 81.20 | 3 | 147.20 | 3 | 209.20 | 12 | 433.40 | 30 |
| 92.80 | 2 | 157.10 | 5 | 234.20 | 3 | 434.40 | 13 |
| 95.10 | 5 | 179.10 | 4 | 237.10 | 3 | 435.30 | 5 |

TS 8/31

ms 9/7/10

USADA 0067

>> Cross Correlation for the spectra below = 0.9787 <<



>>> Normalized Tabulation of First Spectrum Printed Above

Result of BIG

Baa

| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 53.10 | 3 | 105.10 | 3 | 155.10 | 3 | 209.20 | 10 |
| 55.20 | 3 | 107.00 | 3 | 169.15 | 3 | 233.20 | 3 |
| 59.05 | 3 | 109.10 | 3 | 182.15 | 3 | 247.20 | 3 |
| 61.00 | 2 | 117.05 | 3 | 191.30 | 4 | 287.25 | 3 |
| 71.90 | 2 | 119.20 | 3 | 193.20 | 6 | 301.10 | 4 |
| 73.10 | 61 | 129.05 | 7 | 194.10 | 3 | 342.30 | 3 |
| 74.20 | 4 | 131.10 | 3 | 195.20 | 4 | 417.30 | 11 |
| 75.10 | 12 | 132.05 | 2 | 196.10 | 5 | 418.40 | 5 |
| 77.15 | 2 | 133.05 | 4 | 197.10 | 3 | 432.40 | 100 |
| 79.00 | 4 | 143.10 | 5 | 201.10 | 3 | 433.30 | 31 |
| 91.00 | 5 | 147.30 | 4 | 208.05 | 10 | 434.40 | 20 |

>>> Normalized Tabulation of Second Spectrum Printed Above

Result of BIG

Baa

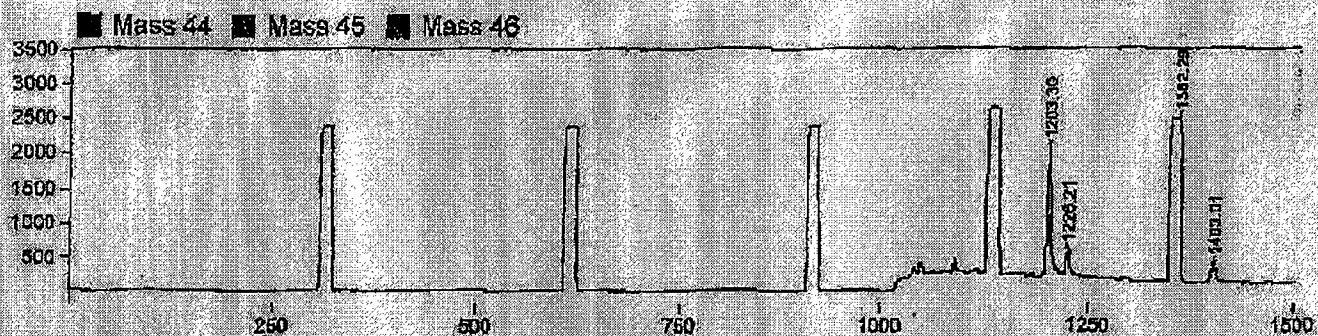
| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 55.10 | 7 | 105.00 | 4 | 180.80 | 4 | 246.80 | 3 |
| 67.20 | 3 | 107.10 | 3 | 183.10 | 4 | 301.10 | 3 |
| 68.60 | 2 | 115.05 | 4 | 193.30 | 8 | 302.50 | 3 |
| 70.20 | 4 | 128.10 | 2 | 195.10 | 4 | 327.30 | 3 |
| 72.00 | 4 | 129.10 | 4 | 196.20 | 6 | 417.20 | 11 |
| 73.10 | 65 | 130.00 | 2 | 199.20 | 2 | 418.30 | 6 |
| 74.10 | 5 | 131.10 | 4 | 207.20 | 3 | 431.20 | 4 |
| 75.13 | 4 | 143.10 | 3 | 208.20 | 12 | 432.40 | 100 |
| 81.20 | 3 | 147.20 | 3 | 209.20 | 12 | 433.40 | 30 |
| 92.80 | 2 | 157.10 | 5 | 234.20 | 3 | 434.40 | 14 |
| 95.10 | 5 | 179.10 | 4 | 237.10 | 3 | 435.30 | 5 |

TS 8/31

ms 07/10

UCLA Olympic Analytical Laboratory

| AS | AS Method | Identifier | Comment | Preparation | Post Script | Method |
|----|-----------|----------------|---------|-------------|-------------|-------------------|
| X | 52 | >Internal No 9 | 9CS | 10 mL | | method1[dols] met |



| Rt [s] | d 13C/12C [per mil] vs. VPDB |
|--------|------------------------------|
| 1203.3 | -32.736 |
| 1226.2 | -31.737 |
| 1403.0 | -26.417 |

DEUTSCHE SPORTHOCHSCHULE
KÖLN
INSTITUT FÜR BIOCHEMIE
IOC AKKREDITIERTES LABOR
FÜR DOPINGANALYTIK

Prof. Dr. Wilhelm Schänzer * Carl-Diem-Weg 6 * 50933 Köln

Prof. Dr. Wilhelm Schänzer
C/O Deutsche Sporthochschule Köln
INSTITUT FÜR BIOCHEMIE
Carl-Diem-Weg 6
50933 Köln

TELEFON: 0221-4971313
TELEFAX: 0221-4973236

total pages -1-

KÖLN, DEN 8. April 2003

Documentation Analysis Report 202/03

Code: 188702

Decision criterias for GC/C/IRMS results

Based on our reference values the following criterias indicate an application of testosterone or testosterone prohormones:

Difference of the $\delta^{13}\text{C}$ [‰]- values between androsterone and pregnanediol > 2.6 or
difference between the $\delta^{13}\text{C}$ [‰]- values of etiocholanolone and pregnanediol > 3.3


Prof. Dr. Wilhelm Schänzer

| | | | | |
|----|-------|--------|-----|-----|
| | NH | DS/751 | PR | SG |
| S1 | IN | 8/4 | TEC | MED |
| S2 | OUT | | TH | VE |
| S3 | INDEX | | REC | AP |

GC/C/IRMS - Results

26.02.03

Federation:

Event:

Code-Nr.:

188702

Lab-Nr.:

1013/03 A

Sample receipt:

11.02.03

Results:

The ratio testosterone/epitestosterone is higher than 6
(25.3 ± 0.31 , $n=3$); the GC/C/IRMS results indicate an application
of testosterone or testosterone prohormones

For the sample with the code number 188702 the following $\delta^{13}\text{C}$ [‰]- values were obtained:

testosterone metabolites:

etiocholanolone -30.5 ± 0.2 ($n=5$)

androsterone -29.5 ± 0.3 ($n=5$)

internal reference compounds:

11 β -hydroxy-androsterone -20.46 ; -20.17

pregnanediol -21.1 ± 0.1 ($n=3$)

Conclusions

The $\delta^{13}\text{C}$ [‰]-values of the testosterone metabolites indicate the application of testosterone
or testosterone prohormones.

Shah

June 21, 2001

To: Clients of the UCLA Olympic Laboratory

Regarding: Carbon Isotope Ratio measurements, Update 3

This letter is to update you on our carbon isotope ratio method and to explain the wording we use in our current reports.

DIOL ASSAY

Requests: Currently when you request a CIR analysis we perform the "Diol" assay. The procedure is to request a CIR analysis by fax or email and to provide the sample number. We will check the original data on the sample and determine if the analysis is likely to be successful. If not, we will advise you that we do not believe the analysis will be successful and we may suggest an alternative approach. In order to enhance our understanding of the analysis we also ask that you give us all sample numbers of prior samples from the same athlete that were analyzed at UCLA.

The "Diol" assay determines the carbon isotope ratio (delta value) of two diol metabolites of testosterone which we refer to as M1 and M2, and one metabolite of a testosterone precursor (Pdiol). [See the metabolic map attached.] The assay determines the ratio of $^{13}\text{C}/^{12}\text{C}$ for each of these three steroids. The units are usually called "delta units". In addition to the delta values for these steroids, two other types of measurements are calculated. The first is the ratio of the metabolites to the precursor. Two ratios are calculated: M1/Pdiol and M2/Pdiol. The second is the difference between the metabolites and the Pdiol: M1 Pdiol and M2-Pdiol.

Endogenous reference compound: The Pdiol serves as an endogenous reference compound (ERC). Since it is a metabolite of a precursor (see map) in the testosterone metabolic scheme, its delta value does not change when testosterone is administered. In the typical positive case, the delta values of M1 and M2 are low and the delta value of Pdiol is within the normal range. In negative cases, all three diols have similar delta values. The reporting terminology will be: Positive, Negative, or Indeterminate.

A **POSITIVE** report means that the delta values for both M1 and M2 are at least three standard deviation (SD) units less than the mean (average) of a group of 73 normal males, and the delta value for Pdiol is within 3 SD of the mean of normal males. In addition the two ratios (M1/Pdiol and M2/Pdiol) and the two differences (M1-Pdiol and M2-Pdiol) are more than 3 SD from the range of normal values. These criteria are very conservative because all must be met for the sample to be declared positive.

A **NEGATIVE** report means that all three delta values, the two difference scores, and the two ratios are within the normal range.

An **INDETERMINATE** report means that we were not able to obtain definitive data. The most common reasons for this are insufficient sample volume and low concentrations of the steroids. Since there are several criteria for a positive report, it is also possible that a sample will have one ratio and one difference score that are normal and the other ratio and difference score will be abnormal. Other combinations of results are possible. Based on our current understanding of the theory underlying CIR measurements, we believe that in these mixed cases that we classify as indeterminate, some of the molecules of M1 or M2 are derived from pharmaceutical testosterone (or another exogenous steroid which is metabolized to

testosterone) and some of the molecules are from natural (endogenous) testosterone. This is expected when the body contains a mixture of pharmaceutical and natural testosterone and metabolites. This situation is likely to occur at the later times in the curve that relates delta values to time since drug administration. As our clients gain further understanding of the CIR analysis, you may have other opinions about how we report mixed cases.

SAMPLES FROM OTHER LABORATORIES

Occasionally we are asked to perform a CIR analysis on a sample that was originally processed at another sample. We are not comfortable with this for four reasons: 1) we do not know the details of handling and storage of the sample in the other laboratory, 2) we do not have any control over the chain-of-custody until the sample arrives at UCLA, 3) we do not have screening data (T/E, testosterone concentration, etc.) obtained at UCLA therefore we cannot adequately plan the analysis, and 4) we do not have control samples that were handled in the same way. We do not have any reason to believe that the CIR analysis is affected by storage conditions, time factors, or temperature nevertheless we cannot be absolutely certain that our in-house control data applies to such samples. In the future we will decline to perform the CIR analysis unless there is sufficient sample volume to perform both a steroid analysis and a CIR analysis.

ANDRO & ETIO ASSAY

Under certain circumstances we also offer the Andro & Etio assay. The advantages are that it is simpler to perform, less expensive, and it almost always yields definitive results. The disadvantage is that there is no PdIOL to serve as an ERC, therefore we are not able to calculate ratios or difference scores. Most clients seem to prefer the DIOL assay, however we suspect that in the future the Andro&Etio assay will be developed to the point where the lack of an ERC will not be a disadvantage. At this time we recommend the "diol" assay. Occasionally we will advise you that it is not possible to perform the DIOL assay and that the only CIR option is the Andro & Etio assay.

GENDER

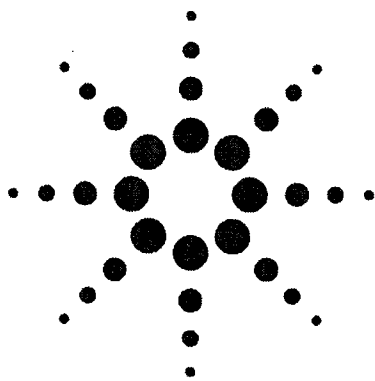
At the present time we do not recommend performing the CIR analysis on samples from females. If you still wish to have the analysis we will determine if it is feasible to proceed based on the existing data from the athlete.

EPITESTOSTERONE

We have been working on a CIR assay for epitestosterone and we have recently presented our findings at a national meeting. There is no peer reviewed publication at this time and it will take about a year to prepare a manuscript and get it published. Nevertheless, at your request we will conduct the epitestosterone analysis on urine samples reported "epitestosterone > 200 ng/ml".

TURN-AROUND-TIME

In an attempt to keep the costs down we are performing the analyses one week per month. Typically this is the fourth week of the month. It takes about a week to complete the analysis, calculate the results, and the issue the report.



Agilent Gas Chromatographs

Fundamentals of Gas Chromatography



Agilent Technologies

Notices

© Agilent Technologies, Inc. 2002

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

Manual Part Number

G1176-90000

Edition

First edition, May 2002

Printed in USA

Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, Delaware 19808-1610 USA

In This Book...

This book contains information for using your gas chromatograph (GC) effectively.

1 What Gas Chromatography Is

This chapter describes gas chromatography—its effects and its uses—and the chromatographic hardware.

2 Injecting Samples

This chapter describes the most common ways of getting the sample into the GC.

3 Separating Components

The column separates the sample into components. This chapter tells how this works and how to use it.

4 Detecting Components

This chapter describes three common GC detectors.

5 Interpreting Chromatograms

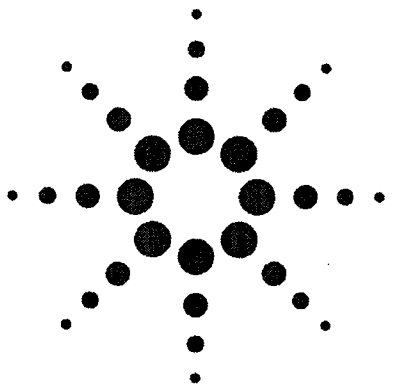
The final chapter discusses how to identify peaks and how to determine the amounts of each component.

Contents

| | | |
|----------|--|----|
| 1 | What Gas Chromatography Is | |
| | A Separation in Time | 10 |
| | The System | 11 |
| | The gas source | 12 |
| | The inlet | 12 |
| | The column | 14 |
| | The detector | 14 |
| | Data reduction | 15 |
| | Instrument control | 15 |
| 2 | Injecting Samples | |
| | Injection Ports | 18 |
| | Packed port | 18 |
| | Split/Splitless port | 19 |
| | Injection technique | 22 |
| | Benefits of automatic injection | 23 |
| | Valves | 24 |
| | Gas sampling | 24 |
| | Liquid sampling | 24 |
| | Inlet Temperature | 25 |
| | Gas samples | 25 |
| | Liquid samples | 25 |
| 3 | Separating Components | |
| | How a Column Separates Components | 28 |
| | The basic principles of chromatography | 29 |

| | |
|-------------------------------------|----|
| Column Types | 30 |
| Capillary columns | 30 |
| Packed columns | 30 |
| Column tubing | 31 |
| Column Characteristics | 32 |
| Column efficiency | 32 |
| Gas control | 33 |
| Column resolution | 33 |
| Column selectivity | 34 |
| Capillary or Packed? | 35 |
| Column Temperature | 36 |
| Isothermal oven | 36 |
| Programmed oven | 37 |
| 4 Detecting Components | |
| Thermal Conductivity (TCD) | 40 |
| How it works | 40 |
| Flame Ionization (FID) | 42 |
| How it works | 42 |
| Electron Capture (ECD) | 43 |
| How it works | 43 |
| 5 Interpreting Chromatograms | |
| Peak Measurements | 47 |
| Retention time | 47 |
| Peak size | 47 |
| Integrators and data systems | 48 |
| Component Identity | 49 |
| Component Amount | 50 |
| Uncalibrated calculations | 50 |

| | |
|-------------------------|----|
| Area and height percent | 50 |
| Calibrated calculations | 52 |
| Normalization | 54 |
| External standard | 55 |
| Internal standard | 56 |



**Agilent Gas Chromatographs
Fundamentals of Gas Chromatography**

1 What Gas Chromatography Is

| | |
|----------------------|----|
| A Separation in Time | 10 |
| The System | 11 |
| The gas source | 12 |
| The inlet | 12 |
| The column | 14 |
| The detector | 14 |
| Data reduction | 15 |
| Instrument control | 15 |

Gas chromatography (GC) is a laboratory technique that separates mixtures into individual components. It is used to identify components and to measure their concentrations.



1 What Gas Chromatography Is

A Separation in Time

Rather than a physical separation (such as distillation and similar techniques), GC creates a time separation.

It does this by passing the vaporized mixture (or a gas) through a tube containing a material that retards some components more than others. This separates the components in time. After detection, the result is a chromatogram (Figure 1), where each peak represents a different component of the original mixture.

The appearance time can be used to identify each component; the peak size (height or area) is a measure of the amount.

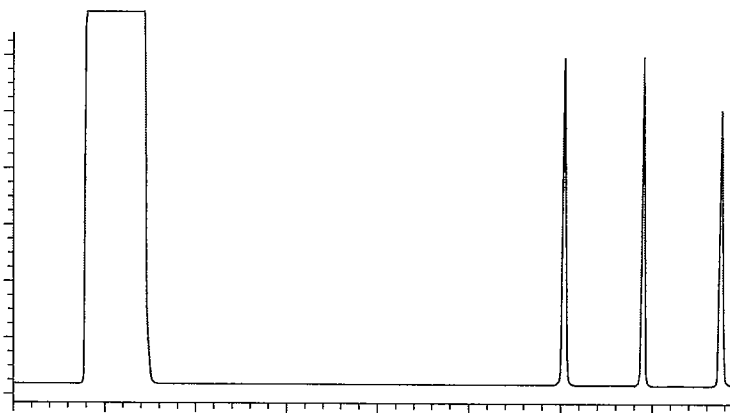


Figure 1 A typical chromatogram

The System

A gas chromatographic system consists of:

- A regulated and purified carrier gas source, which moves the sample through the GC
- An inlet, which also acts as a vaporizer for liquid samples
- A column, in which the time separation occurs
- A detector, which responds to the components as they occur by changing its electrical output
- Data interpretation of some sort

This is summarized in Figure 2.

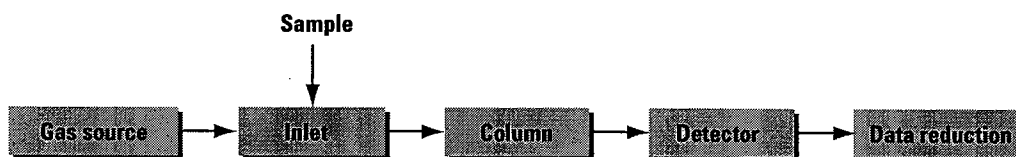


Figure 2 A chromatographic system

The gas source

The carrier gas must be pure. Contaminants may react with the sample or the column, create spurious peaks, load the detector and raise baselines, and so on. A high-purity gas with traps for water, hydrocarbons and oxygen is recommended. See Figure 3.

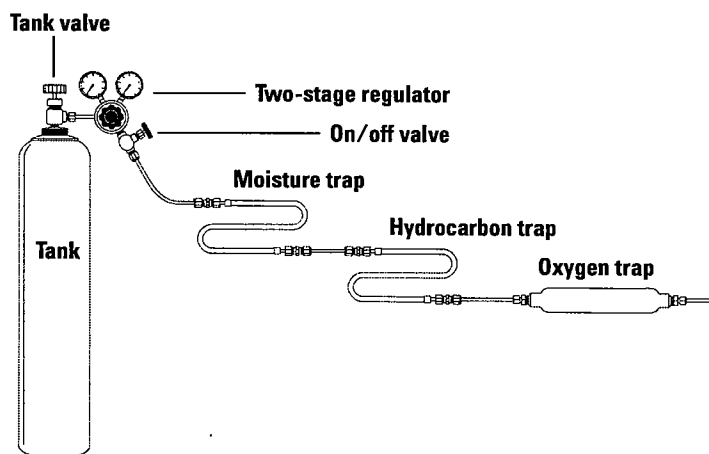


Figure 3 The gas source.

When a house gas supply, rather than separate tanks, is used, have traps for each GC and place them as close to the back of the instrument as possible.

The inlet

The inlet introduces the vaporized sample into the carrier gas stream. The most common inlets are injection ports and sampling valves.

Injection ports

Handle gas or liquid samples. Often heated to vaporize liquid samples. Liquid or gas syringes are used to insert the sample through a septum into the carrier gas stream. The principle (not a real design) is shown in Figure 4.

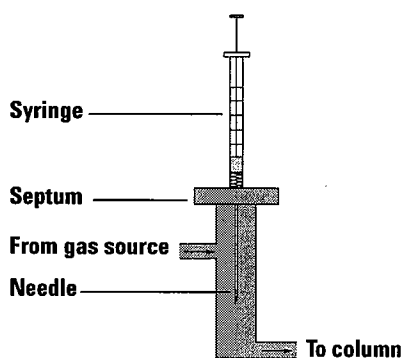


Figure 4 Injection port

Sampling valves

The sample is flushed from a loop which is mechanically inserted into the carrier gas stream. Different valves are used for liquids and gases, because sample volumes are usually quite different. The principle (not a real design) is shown in Figure 5.

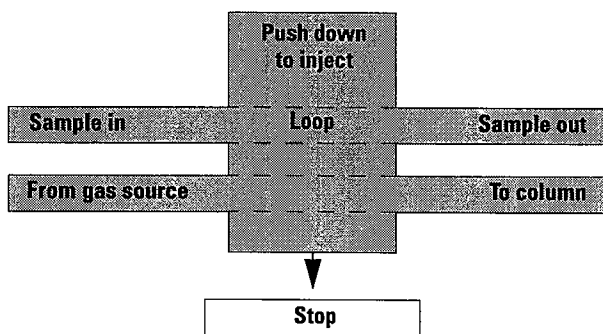


Figure 5 Sampling valve

1 What Gas Chromatography Is

Sample valves are often connected to an inlet, especially the split/splitless inlet in the split mode.

The column

The separation happens here. Because the column type is selected by the user, many different analyses can be performed using the same equipment.

Most separations are highly temperature-dependent, so the column is placed in a well-controlled oven. See Figure 6.

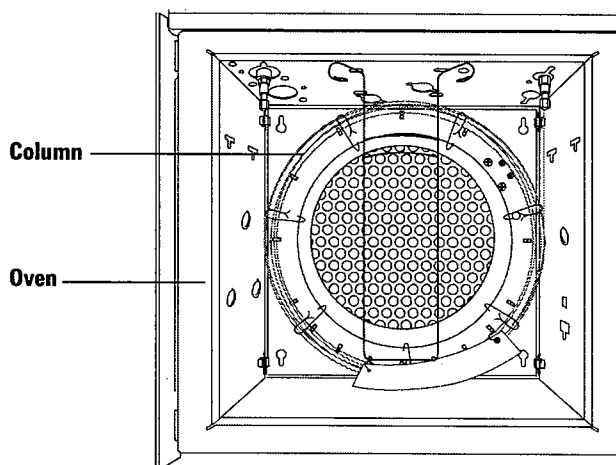


Figure 6 The column and oven

The detector

The gas stream from the column, which contains the separated components, passes through a detector. The output from the detector becomes the chromatogram. See Figure 7.

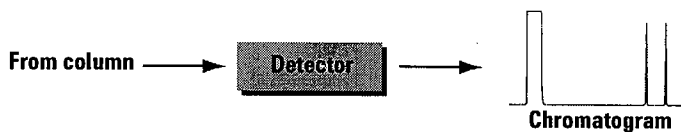


Figure 7 The detector

Several detector types are available, but all perform the same tasks:

- Produce a stable electrical signal (the baseline) when pure carrier gas (no components) is in the detector.
- Produce a different signal when a component is passing through the detector.

Data reduction

Measurement

The chromatogram leaves the detector as an electrical signal. It can be:

- Recorded on a strip chart recorder
- Processed by a digital integrator
- Processed by a computer-based data system

A strip chart recording must be measured to determine the peak times and sizes. Integrators and data systems perform these measurements directly. They are strongly recommended because of their reproducibility and sensitivity.

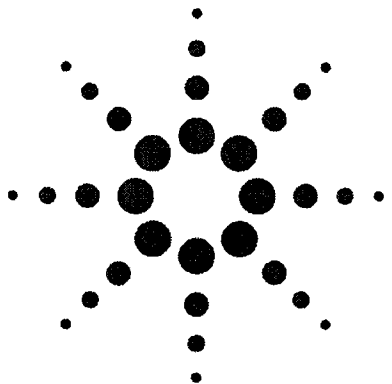
Calculation

The list of times and sizes must be converted to component names and amounts. This is done by comparison to times and responses of known samples (calibration samples). This can be done manually, but for speed and accuracy, a data system is best.

Instrument control

Some data system/GC combinations also provide direct control of the GC by the data system computer. This allows the creation of stored methods, which are invoked as needed, and permits a high degree of analysis automation.

1 What Gas Chromatography Is



2 Injecting Samples

| | |
|---------------------------------|----|
| Injection Ports | 18 |
| Packed port | 18 |
| Split/Splitless port | 19 |
| Injection technique | 22 |
| Benefits of automatic injection | 23 |
| Valves | 24 |
| Gas sampling | 24 |
| Liquid sampling | 24 |
| Inlet Temperature | 25 |
| Gas samples | 25 |
| Liquid samples | 25 |

Some samples are already gases (such as room or outside air, heating gas, etc.) and can be injected directly using either a gas syringe or a gas sampling valve.

Most samples are liquids and must be vaporized in order to be analyzed by gas chromatography. This is usually done with a heated injection port in combination with either a liquid syringe or liquid sampling valve.



Injection Ports

The design and choice of injection ports depends on the column diameter and type. The column types, packed and capillary, are described in the next chapter.

Packed columns and wide-bore capillary columns use the packed port; narrow-bore capillary columns use the split/splitless port.

Packed port

The packed port was developed for packed columns. Removable liners adapt it for the specific column diameter, usually either 1/8- or 1/4-inch. A typical design is shown in Figure 8.

When wide-bore capillary columns appeared, liners were created to allow their use with the packed port. These columns have sample capacities similar to packed columns.

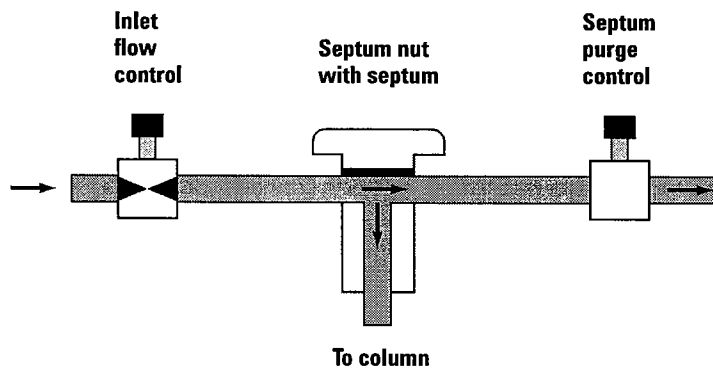


Figure 8 Packed injection port

The sample is injected with a syringe inserted through the septum into the carrier gas stream. The heated port vaporizes it (if it is a liquid) and the carrier gas sweeps it into the column.

Split/Splitless port

The split/splitless port, used with capillary columns, has two operating modes.

Split mode

Capillary columns have low sample capacities. Very small sample sizes, usually much less than a microliter, must be used to avoid overloading the column.

It is very difficult to handle such small sample sizes. The split mode provides a way to inject a normal-size sample, vaporize it, and then transfer only part of it to the column for analysis. The rest is vented to waste.

A typical split/splitless port in *split mode* is shown in Figure 9.

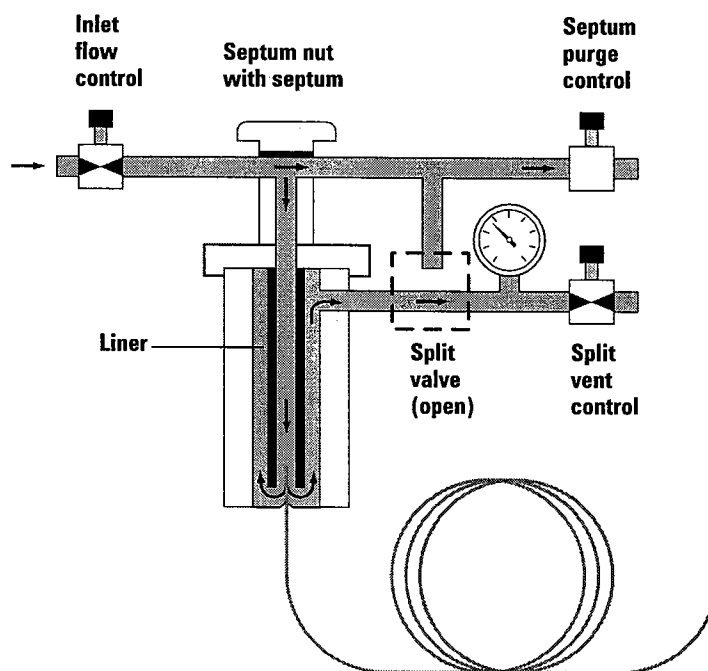


Figure 9 Split mode

2 Injecting Samples

The split valve is open and remains that way. The sample is injected into the liner, where it vaporizes. The vaporized sample divides between the column (high flow resistance) and the split vent (adjustable flow resistance).

Splitless mode

This mode is particularly well suited to low concentration samples. It traps the sample at the head of the column while venting residual solvent vapor in the inlet to waste.

Two steps are involved:

1 Sample injection

Close the split valve. The carrier flow divides between the septum purge and the column. The pressure at the head of the column, and therefore the flow through it, is set by the split vent control.

Inject the sample. The solvent, the major component, creates a saturated zone at the head of the column which traps the sample components.

Figure 10 shows the flows at injection in the *splitless* mode.

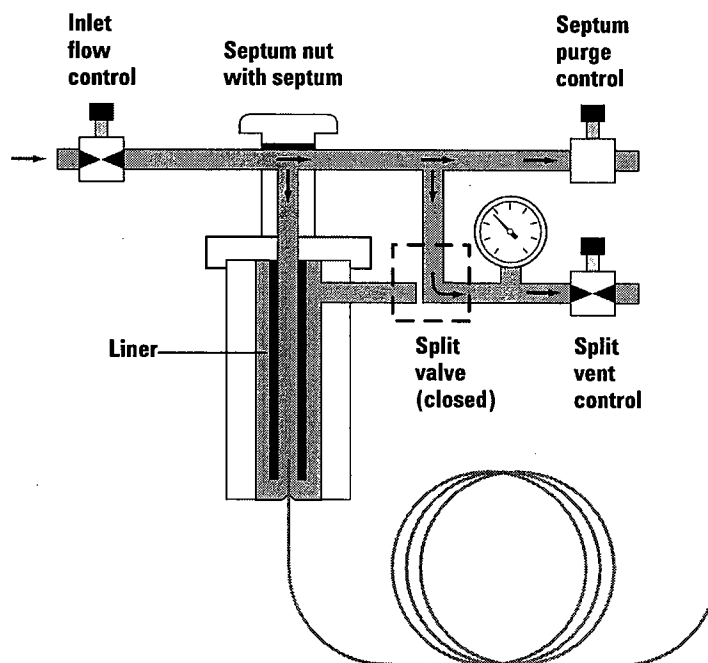


Figure 10 Splitless mode at injection

2 Inlet purge

After the sample has been trapped on the column, open the split valve. The residual vapor in the inlet, now mostly solvent, is swept out the vent.

The flows are now the same as in the split mode (Figure 9).

Raise the oven temperature to begin moving the components through the column.

This works well for components with boiling points higher than that of the solvent. The solvent peak will be large. The oven temperature profile is used to separate peaks of interest from the solvent.

Splitless mode steps

A successful splitless injection consists of these steps:

- 1 Vaporize the sample and solvent in a heated inlet.
- 2 Use a low column temperature to create a solvent-saturated zone at the head of the column.
- 3 Use this zone to trap and reconstitute the sample at the head of the column.
- 4 After all, or at least most, of the sample has transferred to the column, discard the remaining vapor in the inlet by opening the split vent valve.
- 5 Raise the oven temperature to release the solvent and then the sample from the head of the column.

Starting values

You must experiment to find the optimum parameters. Table 1 contains some suggested starting values:

Table 1 Splitless mode inlet parameters

| Parameter | Suggested starting value |
|----------------------------|-------------------------------------|
| Oven temperature | 10°C below solvent boiling point |
| Oven initial time | ≥ Split vent valve open time |
| Split vent valve open time | Liner volume x 2 / Column flow rate |

Injection technique

Each peak begins as part of a region of vaporized sample surrounded by carrier gas. This width of this region broadens by diffusion while the peaks are in the column. No peak can be narrower than the initial region.

Since it is much easier to separate narrow peaks than broad ones, the width of the initial region must be minimized. The ideal injection is:

- 1 Fill the syringe and adjust the amount.
- 2 Push the needle through the injection port septum as far as it will go (port designers assume that you will do this).
- 3 Press the syringe plunger quickly.
- 4 Immediately withdraw the needle from the port.

The important parameter is speed. Any hesitation leads to increased region width.

A skilled operator can achieve 3 to 4% repeatability in sample size, provided he uses the technique described. Mechanical devices that limit the syringe plunger travel can improve on this.

Avoid techniques in which the sample is trapped between two air bubbles. This requires you to make two estimates and doubles the error in sample size.

Benefits of automatic injection

Automatic injectors provide a solution to the injection problem. They make highly reproducible injections. Because of this, they often permit a simpler calculation of peak amount (external instead of internal standard).

If part of an automatic sampler (equipped with a sample tray and connected to a data system), fully automated analyses become possible.

2 Injecting Samples

Valves

Gas sampling

A gas sampling valve consists of a sample loop and a means of shifting it in and out of the carrier gas stream.

A common form of the mechanism is shown in Figure 11.

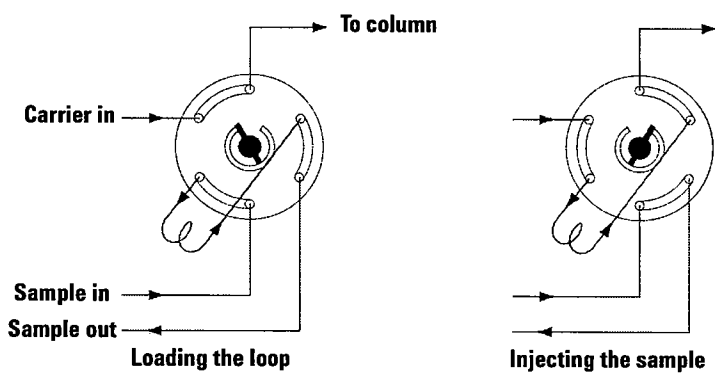


Figure 11 Gas sampling valve

Sample size is determined by the sample loop. This is replaceable, so that a single valve can provide a variety of highly reproducible sample injection sizes.

Liquid sampling

The principle is the same as for a gas sampling valve. Because a liquid sample requires a much smaller volume, the “loop” is part of the valve structure and is not replaceable.

To change sample size, you must replace the entire valve.

Inlet Temperature

Gas samples

For gas samples, the inlet does not have to vaporize anything so the inlet does not have to be heated.

However, most chromatographers prefer to heat the inlet to ensure that nothing condenses in it. A temperature of 100°C is often used.

Liquid samples

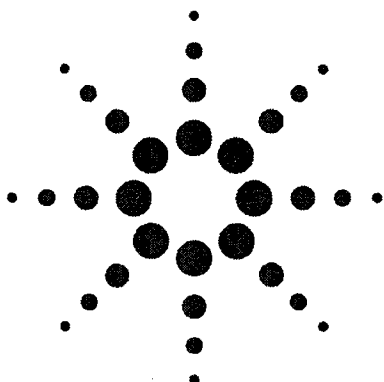
Liquid samples require a heated inlet. The temperature must be high enough to vaporize the sample but not so high that degradation occurs.

Hot enough Start with the solvent boiling point and examine the peaks. If they are all about the same shape (the sizes will differ), the inlet is probably hot enough. If the later peaks show excess broadening, raise the inlet temperature about 10°C to see if the shapes improve.

Too hot If you have more peaks than components and if they are poorly formed, suspect degradation problems.

Degradation in the inlet creates peaks whose size depends strongly on inlet temperature. To detect this, make a second analysis at a slightly lower temperature. Compare the peak sizes; any significant change could indicate degradation in the inlet.

2 Injecting Samples



3 Separating Components

| | |
|--|----|
| How a Column Separates Components | 28 |
| The basic principles of chromatography | 29 |
| Column Types | 30 |
| Capillary columns | 30 |
| Packed columns | 30 |
| Column tubing | 31 |
| Column Characteristics | 32 |
| Column efficiency | 32 |
| Gas control | 33 |
| Column resolution | 34 |
| Column selectivity | 34 |
| Capillary or Packed? | 35 |
| Column Temperature | 36 |
| Isothermal oven | 36 |
| Programmed oven | 37 |

The separation of a mixture into individual components occurs in the column. Many columns are available to separate mixtures. The choice depends on the nature of the mixture and the kind of information desired. However, all columns function using the same basic mechanism.



How a Column Separates Components

This is a cross-section of a column containing a two-component injected sample (the colored dots). There is no packing or coating; the column is just an empty tube (Figure 12).

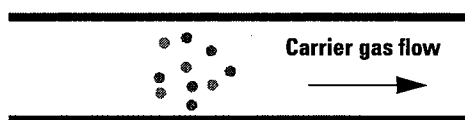


Figure 12 An uncoated column

If we look again a few seconds later, the appearance has changed (Figure 13).

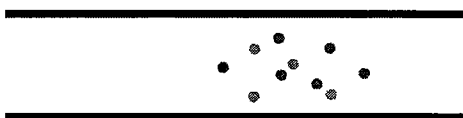


Figure 13 A few seconds later

The "sample" has moved to the right because of the carrier gas flow. It has broadened because of the concentration difference between the sample and the pure carrier gas surrounding it.

The components are still mixed.

Now we add a thin coating of a high-boiling substance on the inside surface of the column and repeat the experiment (Figure 14).

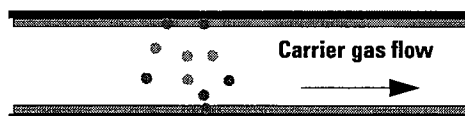


Figure 14 A coated column

We can use any coating we wish. In this case, we select one that dissolves the blue-dot component but not the yellow-dot component.

The blue-dot component distributes itself between the coating and the gas. The yellow-dot component stays in the gas phase.

When we examine the column a few seconds later, we find this: Figure 15.

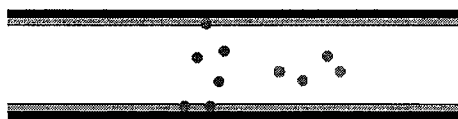


Figure 15 A few seconds later

The yellow-dot component is not attracted to the coating. It moves through the column at the speed of the carrier gas and will emerge first.

The blue-dot component divides its time between the stationary coating and the carrier gas. It travels through the column at a slower speed and will emerge later.

The sample has begun to separate into two peaks.

The basic principles of chromatography

- When a vaporized component is presented with a gas phase and a coating phase, it divides between the two phases according to its relative attraction to the two phases.
- The “attraction” can be solubility, volatility, polarity, specific chemical interaction, or any other property that differs from one component to another.
- If one phase is stationary (the coating) and the other is moving (the carrier gas), the component will travel at a speed less than that of the moving phase. How much less depends on the strength of the attraction.
- If different components have different “attractions”, they will separate in time.

Column Types

Capillary columns

A capillary column is an open tube with the stationary phase coated on its inside surface. There is no packing.

These columns range from about 0.1 to 0.5 mm inside diameter. A typical column length is 30 m. See Figure 16.

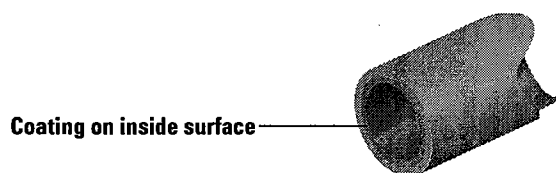


Figure 16 A capillary column

Capillary columns produce very narrow peaks. This allows the separation of very complex mixtures. For example, a typical automobile fuel yields between 400 and 500 peaks.

These columns, when made with fused silica tubing, are very inert. Difficult samples such as mercaptans, which tail severely on metal or glass columns, separate to the baseline on such columns.

Capillary columns require smaller samples than packed columns. A special inlet, see "Split/Splitless port" on page 19, allows a convenient-sized sample to be divided before it enters the column.

Packed columns

In a packed column, the stationary phase is coated on a finely-divided inert material to maximize its area and minimize its thickness. The coated material is then packed into a metal, glass, or plastic tube. See Figure 17.

Most metal packed columns are either 1/8- or 1/4-inch outside diameter. Glass columns are generally 1/4-inch outside diameter, but the inside diameter varies to produce the equivalent of the two metal column sizes.

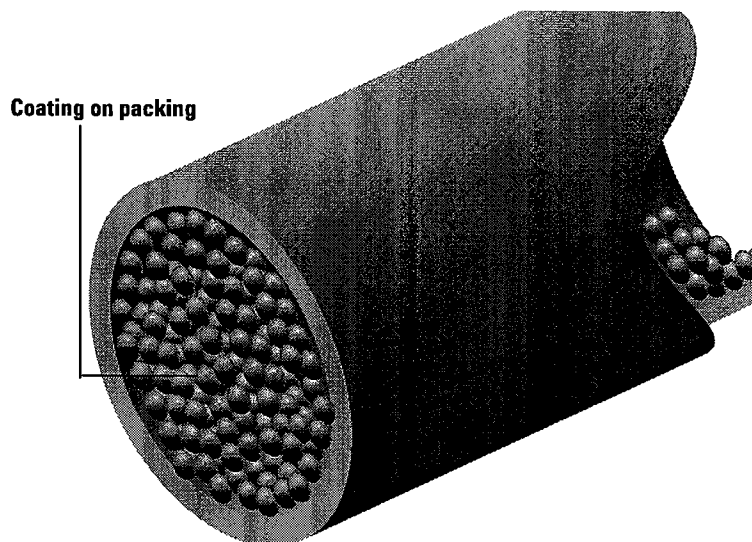


Figure 17 A packed column

Packed columns have high sample capacity, a necessity with older, less sensitive detectors. However, with modern high-sensitivity detectors, this advantage has vanished. Packed columns are still useful for gas samples, but capillary columns offer better resolution for most liquid samples.

Column tubing

Possible tubing materials include:

- Stainless steel—durable, but a relatively reactive surface may cause component loss or peak tailing.
- Glass—fragile, and usually requires treatment to deactivate the surface.
- Fused silica—used only in capillary columns, inert and robust, the preferred material for most uses.

Column Characteristics

The purpose of a column is to produce narrow, well-separated peaks from a multi-component sample. These two purposes are not entirely separate.

Column efficiency

A high-efficiency column produces narrow peaks. See Figure 18.

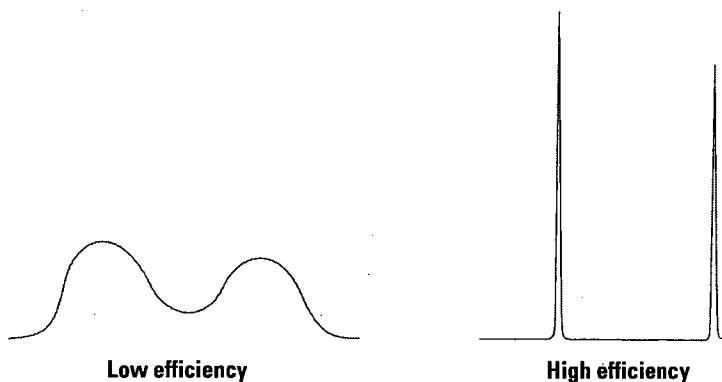


Figure 18 Column efficiency

Efficiency is determined by the column construction (small tubing diameter and thin stationary phase layer is best) and by the carrier gas flow rate.

See Table 2 for recommended flow rates.

Table 2 Recommended carrier flow rates

| Type | Diameter | Carrier flow rate, mL/min | | |
|-----------|-------------|---------------------------|------------|-------------|
| | | Hydrogen | Helium | Nitrogen |
| Packed | 1/8-inch od | 30 | 30 | 20 |
| Packed | 1/4-inch od | 60 | 60 | 50 |
| Capillary | 0.05 mm id | 0.2 to 0.5 | 0.1 to 0.3 | 0.02 to 0.1 |
| Capillary | 0.1 mm id | 0.3 to 1.0 | 0.2 to 0.5 | 0.05 to 0.2 |
| Capillary | 0.2 mm id | 0.7 to 1.7 | 0.5 to 1.2 | 0.2 to 0.5 |
| Capillary | 0.25 mm id | 1.2 to 2.5 | 0.7 to 1.7 | 0.3 to 0.6 |
| Capillary | 0.32 mm id | 2 to 4 | 1.2 to 2.5 | 0.4 to 1.0 |
| Capillary | 0.53 mm id | 5 to 10 | 3 to 7 | 1.3 to 2.6 |

In each range, the lower value is close to optimum for the carrier gas and column combination. The higher value speeds the analysis without sacrificing very much efficiency.

Even higher flows, above the range given, can be used when separation is great or a shorter column is used. Flows below the range given increase analysis time and may cause an abrupt loss of efficiency.

Gas control

Flow in packed columns is usually controlled using mass flow controllers. Capillary columns, because of the very low flow rates, are usually pressure-controlled.

Some GCs provide electronic pneumatic control (EPC). Such instruments allow setting flows from a keyboard and reading them on a display.

Column resolution

A high-resolution column separates peaks down to the baseline. This is much easier if the peaks are narrow (the column is efficient).

A small change in flow rate can have an appreciable effect on resolution.

Combining the mathematical definitions of efficiency and resolution yields an important result:

Column resolution is proportional to the square root of column length.

This means that increasing column length is *not* an effective way to improve resolution. Doubling the column length doubles the analysis time (and the column cost) but only increases resolution by about 40%.

Column selectivity

This is a less clearly defined property of the stationary phase. Essentially, it is how well a phase differentiates between two compounds. Low selectivity—they elute together. High selectivity—the peaks separate.

Capillary or Packed?

Both have their places. Here are some of the considerations.

- Gas analyses are usually done using packed columns. They have the sample capacity to accommodate the rather large gas samples. Common packings for gas analysis include:
 - Molecular sieve—oxygen, nitrogen, helium, hydrogen, CO₂, CO, methane, etc.
 - Alumina—propane and up.
 - Porapaks—ethane, butane, CO₂, etc.

Some, but not all, of these can be used in capillary columns.

- Capillary columns have higher resolution than packed columns. Even with little selectivity, an adequate separation is often obtained.
- One capillary column can perform a variety of analyses that might require a collection of several packed columns to achieve.
- Useful stationary phases for both capillary and packed columns include:
 - Methyl silicones—non-polar to moderately polar
 - Phenyl methyl silicones (5 to 50% phenyl)—olefins, aromatics, to moderately polar
 - Carbowax (polyglycol)—acids, very polar
- The high resolution of capillary columns often permits trading resolution for time. Since resolution depends on the square root of length, an excellent capillary column can be cut into two very good capillary columns with only a minor loss in resolution. Analysis time is reduced to one-half!

Column Temperature

The stationary phase (coating) in the column has a preferred temperature range.

- The *minimum* temperature is usually a melting point. Below this, you are doing gas/solid chromatography; above it, you are performing gas/liquid chromatography. Results can be quite different.
- The *maximum* temperature is usually related to a boiling or degradation point.

Columns are mounted in a temperature-controlled oven because separations are highly temperature dependent.

The oven temperature can be isothermal or programmed. See Figure 19.

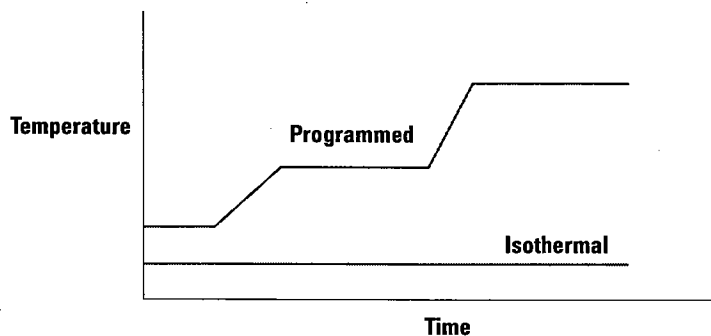


Figure 19 Oven temperature

Isothermal oven

This is the simplest way to run the oven. The oven remains at the same temperature throughout the analysis. It has *advantages*:

- The oven is always ready for a sample analysis.
- There is no recovery time between analyses.

And *disadvantages*:

- Samples with a wide range of component times take a long time to run.
- Because peaks broaden with time, later peaks may be difficult to detect or measure.

Programmed oven

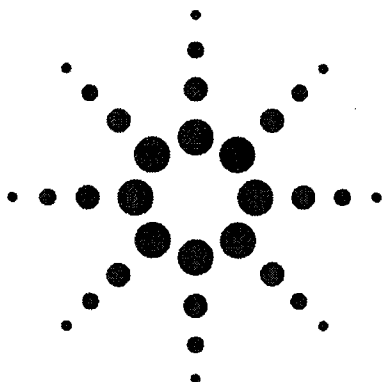
The oven temperature changes, usually upward, during the analysis. The *advantages* are:

- Analysis time is reduced.
- Peak shapes are constant throughout the run, making detection and measurement easier.

The *disadvantages* are:

- Components are subjected to higher temperatures than with an isothermal oven. This could cause degradation of sensitive components.
- The oven must cool to the starting temperature between runs. This cancels part of the time gained.

3 Separating Components



4 Detecting Components

| | |
|----------------------------|----|
| Thermal Conductivity (TCD) | 40 |
| How it works | 40 |
| Flame Ionization (FID) | 42 |
| How it works | 42 |
| Electron Capture (ECD) | 43 |
| How it works | 43 |

The three detectors described in this chapter do most of the GC work. They are augmented by others (see Table 3), mostly element-specific or mass-selective, which are not described in detail.

Table 3 Some other detectors

| Name | Uses |
|------------------------------------|---|
| Nitrogen-Phosphorus Detector (NPD) | Nitrogen- and phosphorus-containing compounds |
| Flame Photometric Detector (FPD) | Sulfur- and phosphorus-containing compounds |
| Atomic Emission Detector (AED) | Tunable for many elements |
| Mass Selective Detector (MSD) | Identify components from mass spectra; when combined with GC, the most powerful identification tool available |



Thermal Conductivity (TCD)

All gases conduct heat, but hydrogen and helium are, by far, the best thermal conductors (see Table 4). When either of these is used as the carrier gas, anything else that may be present causes a decrease in the thermal conductivity of the gas stream.

This change can be measured and used to create a chromatogram.

Table 4 Thermal conductivities of gases relative to hexane

| Gas | Relative thermal conductivity |
|----------------------|-------------------------------|
| Carbon tetrachloride | 0.44 |
| Benzene | 0.88 |
| Hexane | 1.00 |
| Argon | 1.04 |
| Methanol | 1.10 |
| Nitrogen | 1.50 |
| Helium | 8.32 |
| Hydrogen | 10.68 |

Since the TCD operates on thermal conductivity differences, it is clear that hydrogen or helium are the preferred carriers.

How it works

When a voltage is applied to a filament, it heats up. The steady-state temperature depends on the applied voltage, the resistance of the filament, and the rate at which the filament loses heat to its surroundings.

If a filament is immersed in a gas stream, any change in the thermal conductivity of the gas causes a change in filament temperature. This changes the resistance of the filament.

Early TCD designs used four filaments connected as a Wheatstone bridge. The column effluent flowed over two opposite filaments; pure carrier gas (the reference) flowed over the other two. When a component appeared, the bridge became unbalanced.

A modern TCD design is shown in Figure 20.

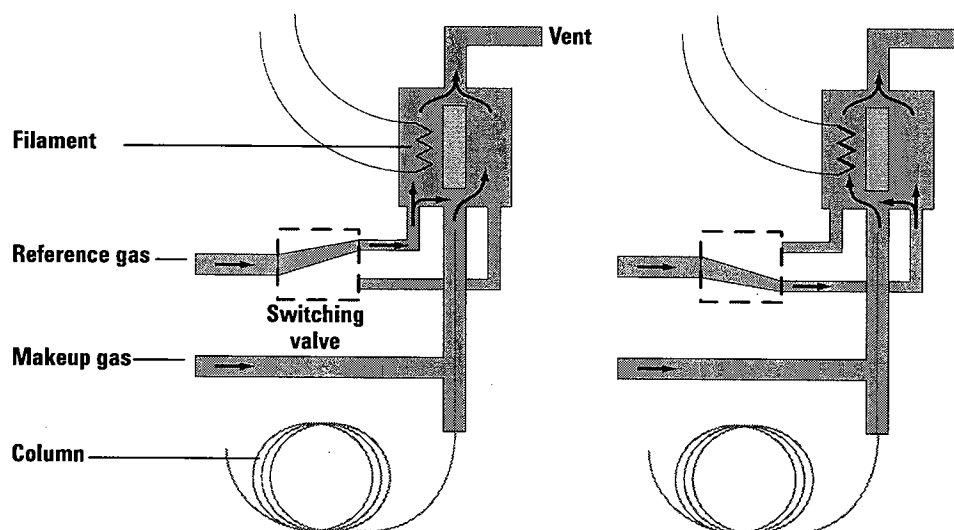


Figure 20 Thermal conductivity detector

This detector uses a single filament. A rapid switching valve causes it to sample the carrier effluent and a reference gas alternately. If the two gas streams are identical—no component present—the filament resistance does not change when the gases switch.

However, when a component enters the detector, the filament temperature drops when the column flow is switched in and then recovers when the reference gas is switched in. The electronics senses this change and adjusts the power to the filament to keep the temperature constant.

The power demand curve amplitude depends on the thermal conductivity difference between the column flow (when a component is present) and the reference gas.

Flame Ionization (FID)

An air/hydrogen flame creates very few ionized particles. However, if a carbon-containing material enters the flame, ion production increases.

How it works

The carrier gas from the column mixes with hydrogen and is burned in air. The FID uses two electrodes, one of which is often the jet where the flame burns, and a polarizing voltage to collect the ions from the flame.

When a component appears, the collected current rises. After amplification, the current creates the chromatogram.

The FID responds to anything that creates ions in a flame, which is essentially all organic compounds (there are a few exceptions).

A general FID design is shown in Figure 21.

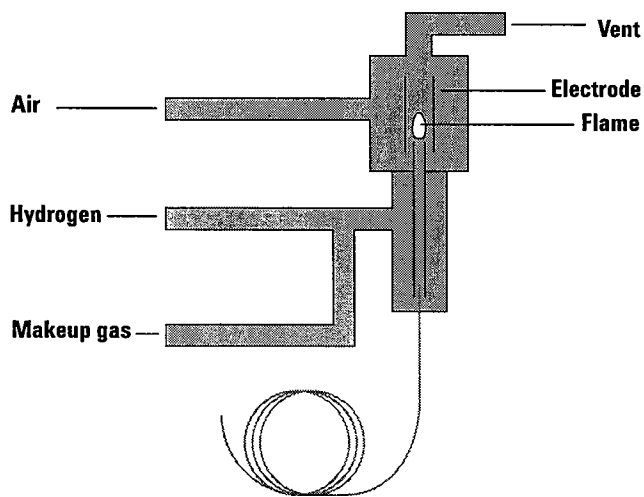


Figure 21 Flame ionization detector

Electron Capture (ECD)

The electron capture detector has found wide use in environmental work because of its very high sensitivity to halogen-containing components, which include most herbicides and pesticides.

How it works

A radioactive isotope, usually ^{63}Ni , in the detector cell emits beta particles. These collide with carrier gas to create showers of low-energy free electrons. Two electrodes and a polarizing voltage collect the electrons as a current.

Some molecules can capture low-energy electrons to form negative ions. When such a molecule enters the cell, some of the electrons are captured and the collected current decreases. After processing, this signal creates the chromatogram.

One form of the ECD is shown in Figure 22.

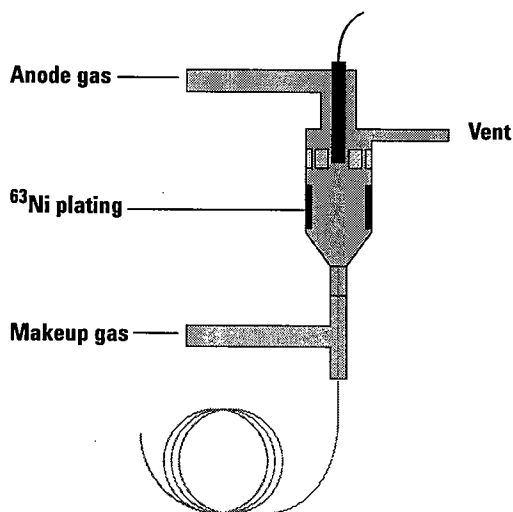


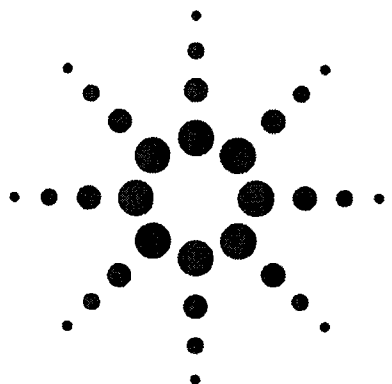
Figure 22 Electron capture detector

4 Detecting Components

The ECD is quite specific, responding strongly to anything that captures electrons and poorly to everything else. Some relative responses are listed in Table 5.

Table 5 ECD sensitivities to selected compounds

| Compound | Response relative to benzene = 1 |
|----------------------|----------------------------------|
| Benzene | 1 |
| Toluene | 3 |
| Acetone | 8 |
| 2,3-Butanedione | 800,000 |
| n-Butanol | 17 |
| Chlorobenzene | 1,200 |
| Bromobenzene | 7,600 |
| 1-Chlorobutane | 17 |
| 1-Bromobutane | 5,000 |
| 1-Iodobutane | 1,500,000 |
| Chloroform | 1,000,000 |
| Carbon tetrachloride | 6,600,000 |



5 Interpreting Chromatograms

| | |
|---------------------------|----|
| Peak Measurements | 47 |
| Retention time | 47 |
| Peak size | 47 |
| Component Identity | 49 |
| Component Amount | 50 |
| Uncalibrated calculations | 50 |
| Area and height percent | 50 |
| Calibrated calculations | 52 |
| Normalization | 54 |
| External standard | 55 |
| Internal standard | 56 |



5 Interpreting Chromatograms

The chromatograph produces a signal that varies with time. When plotted, it produces the familiar chromatogram (Figure 23).

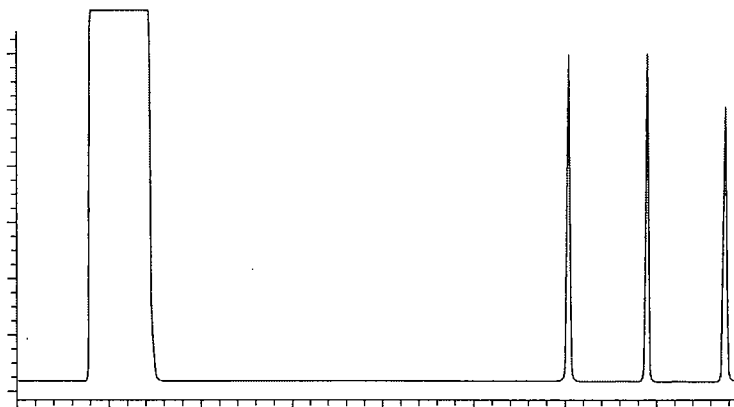


Figure 23 A typical chromatogram

The chromatogram can be converted into a list of peak times and sizes by either manual or electronic means.

Peak Measurements

Two basic measurements can be made on a peak:

- The time after injection when the peak is detected
- The size of the peak

Retention time

The *appearance time*, measured from injection to detection, is the sum of two parts:

- The *plumbing time*—how long it takes for the carrier gas to pass through the column. It is measured by injecting air or some other non-interacting substance.
- The *retention time*—the additional time caused by the component's interaction with the stationary phase in the column.

For most purposes, the plumbing time is ignored and the retention time is taken as the appearance time.

Peak size

Size can be measured either as peak area or peak height, both measured relative to a constructed baseline.

The baseline under the peak cannot be measured directly. It must be constructed from the baselines on either side of the peak.

This is simple with well-separated peaks. It is much more difficult when peaks are merged, on the trailing edge of a solvent peak, or otherwise less than ideal. For this reason, time spent improving the peak separation is time well spent.

Peak height

This is the simplest measurement, requiring only a ruler. It is the vertical distance from the top of the peak to the baseline.

Peak area

This is the area enclosed by the peak signal and the baseline under it. It is best measured by electronic means.

The two size measurements are shown in Figure 24.

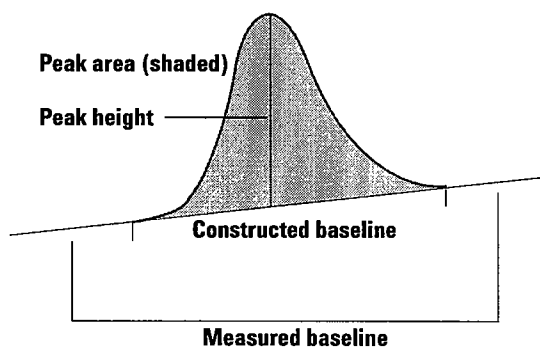


Figure 24 Measuring a peak

Integrators and data systems

Integrators excel at measuring peak areas or heights and peak retention times. They make the conversion of a curve (the chromatogram) into a table (of times and sizes) very simple and reproducible.

Data systems offer the same advantages and considerably more.

- A software integrator is more flexible than that in a hardware integrator.
- Data can be reprocessed using different integration and calculation parameters without re-injecting the sample.
- Systems perform the fundamental calculations described in this chapter, often with more sophisticated features.
- Systems produce user-designed formatted reports.
- Peak calibration becomes a very simple process.
- Both single- and multi-level calibrations are possible.
- Raw and processed data can be archived for later use.
- Systems process data from multiple GCs at the same time.

Component Identity

Because many compounds may elute at the same (or nearly same) retention time, gas chromatography by itself does not usually provide identification of a totally unknown sample.

However, it is a very powerful tool when the problem is more constrained. GC patterns can be compared to identify samples that have a high probability of being the same. For example, crude oil from a tanker can be compared to an oil slick on the ocean to determine if that tanker was responsible for the spill.

GC is quite useful for *eliminating* possibilities. If you know from previous experiment that iso-octane appears at 1.9 minutes, then the unknown peak at 1.5 minutes is definitely *not* iso-octane. But what is it?

Fortunately, you do not have to consider the entire universe of chemical compounds. Sample information limits the list of possibilities. For example, you would not expect to find streptomycin in a paint sample.

When a tentative peak identification has been made, it should be confirmed by repeating the analysis using a column that separates on a different basis. If a component has the right retention time on a boiling point column (methyl silicone) *and* on a polarity column (polyglycol), the identification is probably correct.

GC is especially useful in problems where the expected components are known and quantitation is required. GC will also usually detect the presence of unexpected components (as extra peaks).

Finally, GC can be connected to mass spectrometers or other selective detectors to provide additional data needed for positive identification of unknown components.

Component Amount

Uncalibrated calculations

A detector produces a signal while carrier gas is passing through it. If there is no component at the moment, the signal is the baseline. When a component appears, the signal increases.

The area between the projected baseline and the signal, while the component is passing through, is the peak area. The maximum vertical distance between the signal and the projected baseline is the peak height.

An integrator or data system handles the sometimes very difficult task of drawing the projected baseline, then measures the peak areas and heights. The results are the **Measured Responses (MR)**.

Area and height percent

Each peak is expressed as a percent of the total measured area or height in the run.

The detector is assumed to be equally sensitive to all components. Equation 1 shows the calculation.

$$\text{Amount of peak } n = \frac{[\text{MR of peak } n / \text{Sum of all MRs in the run}] \times 100}{(1)}$$

Advantages

- Fast setup, since no calibration is needed.
- Moderate sample size variation does not affect results.

Disadvantages

- All peaks must be detected.
- Any peaks not detected or not flushed from the column reduce the sum of MRs. This causes overestimation of all measured peaks.

Uncalibrated calculations do not correct for component sensitivity differences. This tends to overestimate the early peaks.

Common uses

- Generating a list of responses and retention times for building a calibration table.
- Analyses where the purpose is fast, reproducible results to be compared with preset limits.
- Useful in process monitoring, product release testing, etc.
- Not useful when absolute accuracy is important.

Calibrated calculations

If Area% and Height% are not adequate, the calibrated calculations use data from standard analyses to create individual peak calibrations.

The simplest calibration is the Response Factor, which is calculated by dividing the known amount of a component by the size of the peak it produces.

Graphically, it is the slope of a plot of component amount versus peak size, as shown in Figure 25.

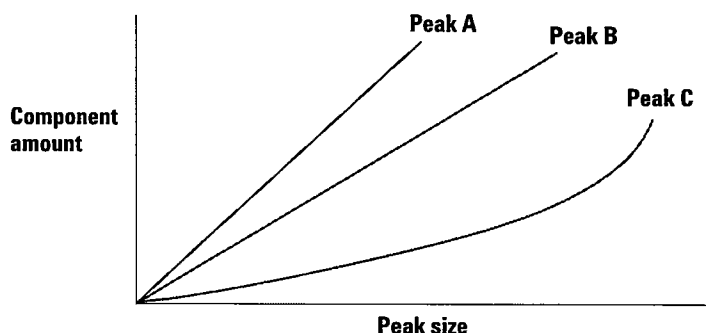


Figure 25 Response factors

Response Factors can be determined by analyzing a single standard mixture containing all of the components to be calibrated.

However, the Response Factor approach makes two important assumptions:

- The amount/size line passes through the origin.
- The amount/size line is straight.

For a trustworthy calibration, both assumptions must be demonstrated experimentally. If the line is really straight and really does pass through the origin, then the response factor is valid.

In Figure 25, Response Factors can be used for peaks A and B, but not for peak C. The two forms of calibration correction are shown in Equation 2 and Equation 3.

For Peaks A and B:

$$\text{CR of peak} = \text{MR of peak} \times \text{Response Factor of peak} \quad (2)$$

For Peak C:

$$\text{CR of peak} = \text{<Response Curve amount> of MR of peak} \quad (3)$$

Peak C can only be corrected by using the entire calibration curve. This is laborious by hand, but is easily done using a data system.

Normalization

The normalization percent is similar to Area% and Height%, but uses Corrected Responses instead of Measured Responses, as shown in Equation 4.

$$\text{Amount of peak } n = \frac{\text{CR of peak } n}{\text{Sum of all CRs in the run}} \times 100 \quad (4)$$

Advantages

- This calculation corrects for component sensitivity differences, which yields more accurate results for early peaks.
- Moderate sample size variation does not affect results.

Disadvantages

- The method must be calibrated.
- All peaks must be detected. Any peaks not detected or not flushed from the column will reduce the sum of CRs. This causes overestimation of all measured peaks.
- All peaks must be identified and calibrated to achieve the highest accuracy. Unknown (and therefore uncalibrated) peaks reduce the absolute accuracy of the calculation.

Common uses

- Provides very accurate results if there are no high-boilers to worry about.

External standard

The great advantage of external standard is that only the peaks of interest need to be calibrated. The calculation is very simple; see Equation 5.

$$\text{Amount of peak } n = \text{CR of peak } n \quad (5)$$

Advantages

- Only the peaks of interest must be calibrated.
- Only the peaks of interest must be eluted and measured.
- Each calibrated peak is computed independently.

Disadvantages

- Peaks of interest must be calibrated.
- The calculation assumes that instrumental drift is negligible. Known check samples must be run regularly to confirm this.
- Constant sample size is essential, since this is an absolute (rather than relative) calculation. This is very difficult to achieve using manual injection. In practice, a gas or liquid sampling valve or an automatic liquid sampler is a necessity.

Common uses

Gas analyses using a sampling valve. As instrument stability improves, and with the help of automatic injection devices to ensure constant sample size, ESTD is taking over many analyses that formerly required ISTD.

Internal standard

Internal standard provides independent calculation of each calibrated peak. It also corrects for variation in sample size, instrument drift, and other factors.

ISTD is considered the most accurate chromatographic calculation, although ESTD with modern equipment is rapidly improving.

The basic calculation is shown in Equation 6.

$$\text{Amount of peak n} = \left[\frac{\text{CR of peak n}}{\text{CR of ISTD peak}} \right] \times \text{Amount of ISTD peak} \quad (6)$$

The quantity **Amount of ISTD peak** is a known amount of the internal standard compound that is added to each sample before analysis.

This is generally considered to be the most accurate of the calculations.

Advantages

- Only the peaks of interest must be calibrated.
- Only the peaks of interest must be eluted and measured.
- Each calibrated peak is computed independently.
- Minor variation in sample injection size does not affect results.
- Minor instrumental drift does not affect results.

Disadvantages

- Peaks of interest must be calibrated.
- A known amount of an internal standard substance must be added to every sample.

Common uses

Liquid sample analysis where high accuracy is required.

Note

The term internal standard has come to have two slightly different meanings:

- 1 ISTD was originally developed to compensate for differences in manual sample injection size. To do this, the internal standard was added to the ready-to-inject sample after any sample workup (distilling, extracting, etc.) was completed. The main requirements for the internal standard were that it not be present in the original sample and that it produce a well-defined peak that is well resolved from the sample peaks. It did not have to be chemically similar to the sample components.
- 2 In many biochemical and related applications, the internal standard is added to the raw sample before sample workup. In this case, it must be chemically similar to the sample so that it will be affected by the workup steps in much the same way. Now the internal standard is being used to correct for two different things: variation in percent recovery during workup and sample size differences in the injection. This is not possible with a single standard. By precisely controlling the sample workup process and experimentally confirming that percent recovery is highly reproducible, that source of error can be reduced to an acceptable level.

5 Interpreting Chromatograms



Agilent Technologies

© Agilent Technologies, Inc.

Printed in USA, May 2002



G1176-90000

Commonly Practiced Quality Control and Quality Assurance Procedures for Gas Chromatography/Mass Spectrometry Analysis in Forensic Urine Drug-Testing Laboratories

B. A. Goldberger

Department of Pathology, Immunology, and Laboratory Medicine
University of Florida College of Medicine
Gainesville, Florida
United States of America

M. A. Huestis

Addiction Research Center
National Institute on Drug Abuse, National Institute of Health
U.S. Department of Health and Human Services
Baltimore, Maryland
United States of America

D. G. Wilkins

Department of Pharmacology and Toxicology
and
Center for Human Toxicology
University of Utah
Salt Lake City, Utah
United States of America

TABLE OF CONTENTS

| | | |
|------|---|----|
| | INTRODUCTION | 60 |
| I. | METHOD VALIDATION | 60 |
| | A. Assay Characterization | 60 |
| | B. Accuracy and Precision of the Analytical Method | 61 |
| | C. Linearity of the Analytical Method | 62 |
| | D. Specificity of the Analytical Method — Interference Studies | 63 |
| | E. Sensitivity of the Analytical Method — Limit of Detection and Limit of Quantitation | 63 |
| | F. Carryover | 65 |
| | G. Other Factors | 65 |
| II. | INSTRUMENT PERFORMANCE | 67 |
| | A. Instrument Checks | 67 |
| | B. Instrument Performance Evaluation and Tuning | 67 |
| | C. Chromatographic Performance | 68 |
| III. | ASSAY CALIBRATION | 69 |
| IV. | QUALITY CONTROL | 70 |
| | A. Required Quality Control Samples | 70 |
| | B. Verification of Quality Control Material | 71 |
| | C. Evaluation of Quality Control Results | 71 |
| V. | CRITERIA FOR DESIGNATING A POSITIVE TEST RESULT | 72 |
| | A. Chromatographic Criteria | 72 |
| | B. Ion Ratios | 72 |
| | C. Mass Spectral Match | 72 |
| | D. Quantitative Result | 72 |
| VI. | MISCELLANEOUS FACTORS | 73 |
| | A. Data Presentation | 73 |
| | B. Dilution Protocols | 73 |
| | C. Reinjection of Extracts | 73 |
| VII. | REVIEW AND EVALUATION OF GC/MS DATA | 73 |
| | REFERENCES | 76 |
| | ABOUT THE AUTHORS | 79 |

Commonly Practiced Quality Control and Quality Assurance Procedures for Gas Chromatography/Mass Spectrometry Analysis in Forensic Urine Drug-Testing Laboratories

REFERENCE: Goldberger BA, Huestis MA, Wilkins DG: Commonly practiced quality control and quality assurance procedures for gas chromatography/mass spectrometry analysis in forensic urine drug-testing laboratories; *Forensic Sci Rev* 9:59; 1997.

ABSTRACT: Forensic urine drug-testing laboratories operate in a prescribed scientific and administrative manner to ensure accurate test results. All specimens positive by an initial immunoassay test must be confirmed by gas chromatography/mass spectrometry (GC/MS). To provide adequate control and verification of these analytical processes, laboratories must implement appropriate policies and procedures to be used in routine practice. This review describes the following topics regarding GC/MS analyses: method validation, instrument performance, assay calibration, quality control, criteria for designating a positive test result, sample and batch acceptance criteria, and GC/MS data review.

KEYWORDS: Accuracy, calibration, carryover, gas chromatography/mass spectrometry, GC/MS, internal standard, laboratory certification, limit of determination, limit of quantitation, linearity, precision, quality assurance, quality control, sensitivity.

INTRODUCTION

Laboratories accredited by the National Laboratory Certification Program of the United States Substance Abuse and Mental Health Services Administration (SAMHSA, formerly the National Institute on Drug Abuse, NIDA), Department of Health and Human Services (HHS) and the College of American Pathologists (CAP) must perform urine drug testing in a prescribed scientific and administrative manner. Testing of specimens under the CAP and HHS Guidelines requires initial testing by an immunoassay, followed by confirmation of all positive initial test results by gas chromatography/mass spectrometry (GC/MS) [70,72].

To provide adequate control and verification of the analytical process, laboratories must implement appropriate policies and procedures regarding GC/MS analysis. This review is intended to discuss the following topics: method validation, instrument performance, assay calibration, quality control, criteria for designating a positive test result, specimen and batch acceptance criteria, and GC/MS data review. Although this review focuses upon those aspects of quality control and quality assurance pertinent to the regulated drug-testing laboratory, many of the components specified below are directly applicable to any laboratory performing drug testing in biological specimens. Indeed, many of the guidelines reviewed below have been adapted from recommended practices for pharmaceutical methods from the Food and Drug Administration (FDA) and the United States Pharmacopoeia (USP),

as well as recommendations resulting from a 1988 Ad Hoc Committee Report to the American Academy of Forensic Sciences (Toxicology Section) and a 1995 Mass Spectrometry and Good Laboratory Practices Workshop organized by the American Society for Mass Spectrometry [9,69,73,74,80].

I. METHOD VALIDATION

A. Assay Characterization

Method development is a process of documenting or proving that an analytical method is acceptable for its intended purpose. For analytical methods to be implemented in laboratory-based regulatory drug-testing programs, the laboratory must be able to demonstrate that the chosen analytical method has the ability to provide *accurate* and *reliable* data. These data can then be used to identify drug presence in a urine specimen according to pre-established administrative reporting limits (cutoff concentrations). Therefore, it is critical that the laboratory identify the key assay characteristics which it will validate prior to implementation of the method into routine use. Also, the laboratory must clearly define the evaluation criteria for each of the key assay characteristics it has selected as part of its validation. It has been suggested that at a minimum, the key assay characteristics to be established and evaluated should include: the accuracy, precision, linearity, specificity, sensitivity, carryover potential, and ruggedness of the analytical method [29]. Additional

characteristics to be evaluated may include: the stability of the analyte under various analytical and storage conditions, identification and concentration of the internal standard(s) for the method, validation of use of partial (diluted) sample volumes, and estimated recovery of the analyte from the matrix [18].

Specific evaluation criteria for method validation generally accepted by the scientific community for an analytical method can vary depending on the particular technique used (i.e., HPLC, GC/MS, LC/MS), as well as its particular application. Much of the available published information with respect to details of validation protocols, such as the number of batches to be evaluated, the number of replicates, and specific acceptability criteria, is based upon chemical analyses performed on autoanalyzers, or HPLC systems, rather than GC/MS systems [2,18,38,41,78]. Therefore, the application of quality control principles to GC/MS analysis of urine specimens has been based largely upon professional consensus, or "generally accepted laboratory practice" in the drug-testing community. This is in some contrast to a formally and experimentally developed, literature-based approach to implementation of quality control principles to a specific technology. Nevertheless, as individual GC/MS methods for drugs of abuse in urine matrices have been developed and published in the literature, quality control principles have been selected and applied in a variety of ways to assist in the validation of methods and increase confidence in the data that are obtained.

B. Accuracy and Precision of the Analytical Method

Two of the most important assay characteristics to be determined during method validation are accuracy and precision. Together, accuracy and precision determine the error of an analytical measurement. Accuracy and precision are frequently considered together because they are interdependent in assessing the acceptability of a method. The *accuracy* of a method, as used in biopharmaceutical or drug-testing analysis, refers to the closeness of the measured value to the true value for the sample. More specifically, it is a measure of the degree to which a mean obtained from a series of experimental observations agrees with the "true" or "accepted" value of the quantity to be measured. *Precision*, on the other hand, refers to the variability of measurements within a set. It is most often used to demonstrate scatter or dispersion between numeric values in a set of measurements that have been determined under the same analytic parameters.

The accuracy and/or precision of an assay can be determined by comparing test results utilizing laboratory-prepared standards and controls with those obtained with an established reference method and/or by analysis of

standard reference materials, such as those available from the National Institute of Standards and Technology (NIST) and CAP [3,17,22,23,66]. Secondary checks may involve reanalyses of performance test specimens and comparison of laboratory results with target means obtained via alternate methods already known to be accurate. (Reanalysis of performance test samples, however, may be prohibited unless the laboratory has obtained prior approval from the submitting agency.)

Accuracy is generally expressed as the percentage difference from the actual value (%DFA) as shown below:

$$\%DFA = [(\text{Mean} - \text{Spiked}) / \text{Spiked}] \times 100$$

An alternate way to determine accuracy is to determine whether the measured mean value is statistically different from the actual value using a *t*-test at 95% confidence [37]. The assessment of accuracy must be carried out on mean values which have been calculated from replicate measurements of reference materials containing known concentration of analyte. At a minimum, triplicate measurements are necessary to establish a single mean value and standard deviation (SD) for any single target concentration. During validation of the assay, it is generally accepted practice to assess accuracy at two to three different concentrations of analyte.

The specific concentrations used for the accuracy evaluation are selected to test accuracy across the range of the standard curve (calibration curve) of the assay. It has recently been recommended that accuracy be assessed using a minimum of 9 determinations over a minimum of three concentrations (e.g., 3 concentrations with 3 replicates each) [65,68]. Other authors have recommended a minimum of 36 determinations over a minimum of 6 concentrations (e.g., 6 concentrations with 6 replicates each) [78].

As stated earlier, the acceptability criteria for accuracy and precision for an assay should be preselected by the laboratory. Generally, accuracy acceptability ranges in forensic urine drug-testing laboratories do not exceed 20% (by convention) of the target concentration. Many laboratories routinely use lower ranges, such as 10%. It should be noted that the acceptable accuracy range selected for initial method validation may differ from that selected for routine use (batch acceptance criteria). For example, a laboratory may require that accuracy be within 10% of the known concentration during method validation, and then choose to increase the acceptable range to 20% for routine daily analysis to accommodate both random and systematic error [38].

Precision of an analytical method is usually assessed in two ways: analysis of multiple measurements during a single analytical run (within-run precision) and analysis

of single, or mean, measurements over many runs (between-run precision). Precision is expressed as the percentage relative standard deviation (%RSD), also referred to as the coefficient of variation (CV), as shown below:

$$\%RSD = [\text{Standard deviation} / \text{Mean}] \times 100$$

Within-run precision can be considered a measure of the precision of an analytical method under optimal conditions. The between-run precision, however, is likely to be a better representation of the precision one might observe during routine performance of the assay because these data are generally subjected to a greater number of sources of variability. The lower the calculated CV, the greater the precision of the assay. Precision of an assay at concentrations below, at, and above the assay cutoff concentration can be determined by repeated analyses of quality control samples on a within- and between-batch basis. One approach to assessing between-run precision of the method is to perform triplicate measurements on three separate concentrations of analyte, across three separate analytical batches. Subsequently, the laboratory evaluates the acceptability of the precision of the method using a criterion selected *a priori*. Generally, within-run and between-run coefficient of variation values of <15% are considered acceptable [2,18,29,33,78]. However, because greater variability is to be expected as analyte concentrations approach the limit of detection (LOD) of the analytical method, the laboratory might choose to increase the acceptability criterion to 20% at its lowest measured concentrations [37].

Finally, an additional technique for evaluation of between-run precision data is to apply a one-way analysis of variance (ANOVA) of the data to ensure that results do not significantly differ between analyses [37,42].

C. Linearity of the Analytical Method

The full range of linearity of a method should be established during initial assay characterization and periodically thereafter with specimens containing drug analytes over a wide range of concentrations. Further, the practical range of linearity, referred to here as the daily linear range, should be documented with every batch based upon data obtained with standards and/or controls [29,65]. Acceptance criteria for evaluating linearity data must include review of chromatographic appearance, retention time, and ion ratio or full-scan spectra matching criteria, for example. Although some analytical procedures may require nonlinear calibration, it is conventional for forensic urine drug-testing laboratories to utilize a linear model and univariate regression for GC/MS analysis. In this model, the independent variable is concentration (X) and

the dependent variable is response (Y), i.e., the value determined by the value of the independent variable. Recommendations for linearity studies are noted below; issues with respect to acceptability of daily assay calibration (range) are considered in a subsequent section of this paper.

In practice, linearity should be established via visual evaluation of a plot of signals (response) as a function of analyte concentration. If a linear relationship appears probable by inspection of such a plot, test results should be evaluated by an appropriate statistical method, such as the method of least squares regression [6,7,29]. Other statistical approaches must be clearly justified by the laboratory. Data from the regression line, such as the correlation coefficient (r), coefficient of determination (R^2), slope, and residual sum of squares, can also provide useful mathematical estimates of the degree of linearity obtained with the analytical method. In addition, since it is not uncommon to expect an increase in variance as a function of concentration, it may be more appropriate to perform a weighted (rather than unweighted) regression analysis to improve accuracy at the lowest concentrations studied [8].

During initial method validation, the laboratory typically analyzes a series of standards (calibrators) that have been prepared at known concentrations of analyte. Data are plotted and analyzed as just described to determine the upper and lower boundaries of linearity. A frequently used criterion for determining the upper and lower boundary limits in the pharmaceutical industry is the point at which the slope of the line deviates from the overall slope by not more than 5% [20]. However, this recommendation is not originally based upon GC/MS analysis. An alternative is to "reverse calculate" the individual concentrations of each standard using the generated regression line and determine whether each is in compliance with the acceptance criteria established for evaluation of quality control samples (e.g., such as $\pm 20\%$ of the target value). Outliers may be identified as those concentrations of analyte at either extreme (high or low) which are outside the 20% criterion. Acceptable linearity, therefore, is demonstrated when the correlation coefficient exceeds a defined value, such as 0.990, and quantitative concentration of each point falls within $\pm 20\%$ of the target value. The discussion regarding evaluation criteria for linearity can be found in Section III.

The laboratory uses this information to establish the range, or concentration interval, which will routinely be used for analysis of samples. Validating the method over a wider range than that used in daily practice provides increased confidence that the routine standard concentrations are well removed from nonlinear response concentrations. If the laboratory elects to perform the linearity

assessment on more than one occasion, a statistical test of linearity can be performed for each standard curve separately using a weighted ANOVA [8,42].

D. Specificity of the Analytical Method — Interference Studies

Specificity refers to the ability of the analytical method to accurately measure an analyte response in the presence of all potential sample components. All methods should at a minimum be investigated for potential interference by endogenous matrix components, as well as common compounds that are structurally similar to the analyte of interest. A complete review of interference studies published regarding forensic urine drug-testing analytes is beyond the scope of this paper; however, some examples are provided below to illustrate general principles.

The potential interference of endogenous urine components with the assay is most frequently assessed by evaluation of urine specimens from several sources (donors) that are known to be drug-free for the analyte of interest. Assessment of interference from structurally related compounds can be determined by fortification of urine with high concentrations (e.g., 1 mg/mL) of potentially interfering analytes and cutoff concentrations of target analytes, or with concentrations of analytes that are expected under therapeutic conditions. For example, possible interference with the measurement of amphetamine and methamphetamine may occur due to the presence of sympathomimetic amines such as ephedrine, pseudoephedrine, phenylpropanolamine, and phentermine [32]. Further, interference with the measurement of morphine and codeine due to the presence of opiate metabolites and synthetic 6-keto-opioids such as dihydrocodeine, hydro-morphone, hydrocodone, oxycodone, and oxycodone has also been described [25].

The determination of potential interferents that are *not* structurally related to the analyte of interest is more difficult to establish. However, the urine drug-testing laboratory may consider evaluating the potential interference of common over-the-counter products, as well as frequently encountered compounds which produce fragment ions also produced by the analyte of interest. In addition, the laboratory may refer to literature reports for interferences experienced by other investigators and assess their method with the potential interfering substances [34,59,67,79]. Although a laboratory cannot be expected to anticipate all potential interferents with its analytical method, it should make its best effort to characterize them whenever feasible.

The problem of interfering substances may be addressed by employing more selective extraction methods,

chromatographic separations, or detection methods. For example, to eliminate potential-false positive amphetamine/methamphetamine results due to the presence of other sympathomimetic amines, aliquots of specimens can be treated with a solution of 0.035 M sodium periodate at room temperature, then subjected to extraction. In the presence of periodate, α -hydroxyamines undergo oxidative cleavage removing the potential interferant [24]. Recent evidence indicates that periodate oxidation should be conducted at pH 7 or lower to prevent possible formation of low levels of amphetamine from extremely high levels of methamphetamine that may be present in the specimen [57]. In addition, lowering the injection port temperature of the gas chromatograph, coupled with other preventative measures, eliminates artifactual production of methamphetamine in the presence of high concentrations of ephedrine and/or pseudoephedrine [24,57,67]. It should be noted that some reported interferences may be method-specific and thus will need to be evaluated by the laboratory on an individual basis, as appropriate.

E. Sensitivity of the Analytical Method — Relationship to Limit of Detection and Quantitation

An analytical method is determined to be *sensitive* if small changes in concentration cause large changes in analytical response. It is directly related to, and frequently defined as, the slope of the standard curve [38]. However, this definition does not account for the variability of a measurement. The limit of detection (LOD) and limit of quantitation (LOQ) are terms which are used to express the ability of the assay to detect small concentrations of analyte, as well as attempting to account for variability of measurement.

The *limit of detection* of a method is the lowest analyte concentration that produces a response detectable above the noise level of the system. The *limit of quantitation* is the lowest concentration of analyte that can be accurately and precisely measured. The LOD and LOQ of a method are dependent upon several factors, such as the electron multiplier voltage, the volume of specimen analyzed, the detector threshold, the type and condition of the chromatographic column, the concentration of analyte, the amount and type of internal standard, the extraction efficiency, and the individual instrument [54]. A significant change or modification to any of these factors will require reassessment of the LOD and LOQ for the method.

There are several approaches for establishing the LOD and the LOQ of an analytical method [1,44,48,65]; at least two of these are routinely used in forensic urine drug-testing laboratories. Recent publications have defined issues of concern regarding LOD and LOQ determi-

nation methods, and have described and compared the two most commonly used methods [1,44].

The first approach to establishing the LOD and LOQ of an assay is based on the measurement of the magnitude of analytical background noise. It is performed by analyzing an appropriate number of blank (drug-free) samples and calculating the standard deviation of these responses. In practice, determining LOD using this approach involves the analysis of negative urine specimens (obtained from at least ten different donors) over time. In this paradigm, the LOD is calculated as the mean of the detected amount or signal intensity plus three standard deviations ($\bar{X} + 3 \text{ SD}$) [48], where 3 is a factor for a 99.9% level of confidence. Similarly, LOQ is calculated as the mean of the detected amount or signal intensity plus ten standard deviations ($\bar{X} + 10 \text{ SD}$). An obvious limitation of this approach is that while this may be an adequate measure of the theoretical LOD of a method, actual concentrations of analyte in biological samples measured at this calculated LOD would be indistinguishable from zero measurements by a large probability [38].

The second commonly employed approach for the determination of LOD and LOQ is based on a signal-to-noise comparison [65]. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. In this context, a minimum of a signal-to-noise ratio of 3:1 or greater is generally agreed to be acceptable for LOD assessment; a signal-to-noise ratio of 10:1 or greater is generally agreed to be acceptable for LOQ assessment. In practice, this approach involves analysis of a series of samples containing low concentrations of analyte.

For GC/MS analysis in urine drug-testing laboratories, SAMHSA defines the lowest analyte concentration that meets signal-to-noise, chromatographic, retention time, and ion ratio or full-scan matching criteria, as the LOD. The lowest concentration, that meets all of the above criteria and quantitates within $\pm 20\%$ of the target concentration and measures within a specified coefficient of variation, is designated as the LOQ.

The first two approaches described above are routinely utilized in laboratories performing regulated urine drug testing, although the second approach is preferred over the first approach since it is based upon measurement of an actual analyte response, rather than the absence of a response. A third approach to determination of LOD and LOQ values is based on the standard deviation of the analyte response and the slope of the standard curve. In this case, the LOD is expressed as 3.3 times the standard

deviation of the response divided by the slope (S) of the calibration curve ($3.3 \times \text{SD}/S$). The LOQ is expressed as 10 times the standard deviation of the response divided by the slope of the calibration curve ($10 \times \text{SD}/S$). The slope is estimated from the standard curve of the analyte, and the standard deviation is estimated by analysis of blank specimens, as described for the first approach. Alternatively, the residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation. It has been recommended that if the detection limit of an assay has been estimated by calculation or extrapolation, such as with the first or third approach described above, this estimate should be subsequently evaluated and validated by the independent analysis of a suitable number of samples known to be prepared near or at the detection limit [65].

The LOD is an important assay parameter due to the use of this value to evaluate retest results. The LOQ is important for defining the minimum accurate quantitative value of the assay. For example, dilutions that produce results less than cutoff but equal to or greater than the LOQ may be utilized with the appropriate dilution factor to calculate test results if the LOQ control included in the batch is found to be acceptable. Dilutions that produce results below the LOQ are not acceptable; specimens must be reassayed at a lower dilution. At least one publication [18] recommends that the assessment of the LOQ be obtained using LOQ samples that are prepared *independently* from that included in the standard curve because the LOQ standard that is included in the standard curve influences the regression equation (and thus is no longer an independent measure). Also, since the LOD and LOQ values are influenced by a variety of factors, such as the individual instrument or detector, it may be advisable for the laboratory to assess these parameters on each instrument used for a particular assay, although a general consensus on this issue has not yet been reached.

Interestingly, there are no requirements and few specific recommendations regarding the number of analyses or analytical runs to be evaluated for LOD and LOQ determination. Suggested numbers of replicates of any single blank or standard range from 10 to greater than 20 [19,55,56]. However, from a statistical standpoint it may be advisable to perform these replicate measurements of each blank or standard, in three separate batches, followed by *t*-tests or one-way ANOVA to determine if the calculated LOD and LOQ values differ significantly over time. A two-way ANOVA may be used to determine if a bias is present between instruments.

Finally, in an effort to reduce interlaboratory LOD and LOQ variation, and to introduce a sample that assesses minimum performance in each confirmation batch,

SAMHSA suggested that the LOQ achieved by the laboratory must be a value equal to or less than 40% of the assay cutoff concentration [71]. To ensure acceptable performance, it is desirable to challenge the limit of sensitivity in each batch by including a quality control sample at this concentration to monitor day-to-day instrument and assay variance.

F. Carryover

The term "carryover" is used to refer to the contamination of a sample by a sample analyzed immediately prior to it [35,63]. In the urine drug-testing laboratory, the term "carryover limit" is used to delineate the concentration of analyte in a sample above which contamination may reasonably be expected to occur. There is at least one common approach to performing such studies that involves the analysis of standards prepared at increasingly higher concentrations of analyte, preferably reflecting the highest concentrations which a laboratory typically encounters during routine analysis of samples. Each standard should be injected separately, followed by injection of a blank or solvent to determine if a signal (response) characteristic of the analyte is present in the sample above a pre-established limit (typically the LOD of the analytical protocol). Once the concentration at which carryover occurs is determined, the laboratory establishes its carryover limit at the next lowest concentration which *does not* have evidence of carryover in the blank or solvent. More precisely, upon completion of carryover studies, a laboratory should define the range of analyte concentrations at which carryover does not occur.

The laboratory should also ensure that the quantitative value for the carryover limit established in the carryover study falls within the linearity of the assay to ensure that the quantitative value is accurate. It is also advisable to evaluate carryover of an assay on each instrument system, including autosamplers, on which the method is to be performed, although there is no general consensus on this issue. This is to ensure that the established carryover limit is properly applied to data obtained on each system routinely used in the laboratory.

To minimize potential carryover, one or more of the following approaches can be utilized:

1. Use extensive solvent wash procedures between injections.
2. Inject solvent between all subject specimens.
3. Dilute the specimen prior to extraction.
4. Periodically determine or reassess the minimum carryover concentration.
5. Assay specimen extracts in ascending concentration order (according to initial immunoassay test results).
6. Reinject all highly concentrated specimens followed

- by solvent blanks or negative quality control samples.
7. Assay a carryover standard followed by a solvent blank or negative quality control sample with each batch to assess carryover at the time of testing.
8. Frequently monitor the level of solvent available in the rinse vial for the autosampler to ensure that a sufficient quantity is available for the entire run.

It is important that criteria be established for evaluating the acceptability of solvent blanks or negative quality control samples that have been inserted to assess possible carryover [80]. If carryover is suspected, a potentially contaminated specimen should be re-extracted, rather than reinjected, because the extract vial may have already been contaminated.

G. Other Factors

Other factors, such as selection of a derivatizing reagent, selection of the internal standard(s) for the assay, selection of ions to monitor for selected-ion monitoring or full-scan analysis, stability of the analyte under various storage conditions, estimation of recovery of the analyte from the matrix, and evaluation of the ruggedness of the analytical protocol, should also be determined during the validation of an analytical method [10,18,29,37,38,78].

Selection of a *suitable derivative* is a critical component of assay development and method validation. There are at least three major reasons for using a derivatized compound. First, the analyte can often be made sufficiently volatile to allow its introduction to the mass spectrometer by gas chromatography, permitting optimal separation of the analyte from possible interfering substances. This, in turn, usually increases the specificity, precision, and sensitivity of the assay. Second, the stability of the analyte during storage, isolation, and thermal volatilization can be enhanced via formation of the derivatized product. Third, the increase in molecular mass resulting from derivatization may be beneficial, providing ions which, by virtue of their higher mass, are more specific for the analyte [28,43]. In cases where two or more derivatives are possible, each should be tested to assess its stability, chromatographic peak shape, and mass spectral properties in the biological matrix. The ideal derivatization procedure should be convenient and rapid to perform, form a consistent and stable product in high yield, require small volumes, be selective for the analyte of interest, be safe to handle, and should not form by-products that interfere with the analysis [4,5,39,40,52].

The selection of a *suitable internal standard* is highly linked to the appropriate selection of a derivative for the assay (if necessary), as well the particular ions to be monitored for analyte identification and quantitation. An

ideal internal standard behaves identically to the analyte throughout the extraction, chromatographic separation, and ionization processes. Stable isotope internal standards appear closest to meeting these criteria. The isotope label exerts only a slight effect on the physical properties, yet the higher mass of the isotope-labeled ions of the internal standard permits them to be readily distinguished from analyte ions by the mass spectrometer [16,26,50,58]. Deuterium-labeled analogs [^2H] are most frequently used as internal standards in urine drug-testing laboratories; other isotope-labeled analogs, such as ^{13}C or ^{15}N , are not commonly used.

Several factors are important to consider when selecting the deuterium-labeled internal standard to be used in an assay. The isotope should not undergo exchange under any of the conditions under which it will be used, such as the extraction, derivatization, or chromatographic separation procedures, as well as at the mass spectrometer's ion source [26]. In addition, the isotope must be stable under routine storage conditions, so that exchange of deuterium and hydrogen does not occur [50]. The isotopic variant selected should have a molecular weight three or more mass units greater than the unlabeled compound because the naturally occurring heavy isotope content of organic compounds in general produces ions of significant intensity at one or two mass units above each carbon-containing compound in the analyte's mass spectrum [26,45-47]. It is therefore critical that the isotopic variant is of high purity (>99%) to prevent interference with the analyte of interest during the analysis. Also, the labeling should be in such a manner that the isotopic atoms are located in proper molecular structure so that, after the fragmentation or ionization process, a sufficient number of high-mass ions that retain the label are present in significant intensities and will not interfere with the intensity measurement of the corresponding ions derived from the analyte [46].

The laboratory must carefully evaluate the concentration of internal standard used in its assay to ensure that there is no contribution to analyte signal itself. In effect, the substance ratio of internal standard material to analyte should be selected to give the least imprecision of quantitative analysis and to afford equal ion signal responses during mass spectrometric analysis of the analyte. Under certain conditions, improved sensitivity may be observed by the addition of a large excess of isotopically labeled analogue to reduce adsorptive losses ("carrier effect"). However, this approach is not generally preferred in urine drug-testing laboratories, where a specific administrative cutoff value must be applied. When excess deuterated internal standard is used, analytical precision usually suffers [43], which would not be desirable when a specific quantitative cutoff is needed. Therefore, it is recom-

mended that the laboratory eliminate or minimize adsorptive losses, rather than add excessive amount of internal standard.

The choice of *ion or ions to be monitored* for GC/MS assays has an important influence on analytical specificity. Generally, ions of high, even mass-to-charge ratios have fewer possible origins and are therefore more likely to be characteristic of a particular analyte. The laboratory initially performs a preliminary ion selection based on full-scan mass spectrometric analysis. Ions of high mass-to-charge ratios and good intensity are the first choice for use in routine assays. It is recommended that laboratories using selected-ion monitoring utilize at least three characteristic ions for the analyte of interest, and a minimum of two characteristic ions for the internal standard [72].

Laboratories using full-scan GC/MS must identify those ions with sufficient signal intensity and high-mass to use for qualitative identification of the compound ("matching criteria"). The laboratory should be able to demonstrate that the full-scan spectra it achieves, and plans to use on a routine basis, is stable and reliable over time. Further consideration of these requirements is discussed in Section V.

While most forensic urine drug-testing laboratories are using electron impact ionization (EI) for GC/MS assays, chemical ionization (CI) MS may also be utilized as the mode of detection to improve assay sensitivity and specificity. Chemical ionization MS typically produces an intense molecular ion and only a few fragment ions; therefore, one or two analyte ions may be monitored. A further discussion of these two methods of ionization are beyond the scope of this paper; however, the use of CI is acceptable only if the selectivity, accuracy, and precision of the CI process and method have been fully evaluated [26,81].

Another important parameter to assess during the method validation phase is that of *stability of the analyte*. This includes stability of stock solutions of analyte as well as stability of the analyte in biological matrix. Stability studies will typically be performed to assess stability under different temperatures (storage conditions) and different lengths of time (in-process stability and long-term stability). Stability of the analyte can be assessed at room temperature, refrigerated, and frozen storage conditions. The length of time under each storage condition to be evaluated can range from days to weeks. It is recommended that the laboratory at least establish analyte stability under its own anticipated storage and processing conditions. Although there are many different approaches to the performance of stability studies, a common approach is to use quality control materials prepared at known concentrations to assess stability [18].

Recovery of the analyte from a biological matrix must be determined to ensure that it is adequate and consistent. It is recommended that recovery studies be performed across the range of the standard curve, preferably at the lowest, mid-range, and highest concentrations encompassed by the curve [18,61,78]. Typically, a set of samples of known concentrations is prepared in triplicate at three different concentrations, internal standard is added, and the sample then extracted, derivatized, and analyzed by the GC/MS procedure. A second set of samples is also concurrently analyzed; however, the internal standard is not added until just prior to derivatization. Recovery is then calculated by comparing the calculated concentrations of the two sets of samples and expressing total recovery of the method as a percent. Intermediate points in the extraction process may also be evaluated.

The *ruggedness and reliability of the assay* should be established. Critical assay steps need to be identified, including assessing the importance of pH, solvent mixtures, derivatizing reagents, and temperature and incubation times utilized during the hydrolysis and derivatization processes. In addition, the level of expertise required to perform the analysis needs to be assessed. It must be determined whether the assay can be stopped and restarted, and how long the derivatized analytes are stable. A system for monitoring assay performance variables such as the number of rejected batches, calibration curve parameters (including slope, y-intercept, and correlation coefficient), and quality control results must be established [42,65,78].

II. INSTRUMENT PERFORMANCE

A. Instrument Checks

In addition to developing validated assays for the purpose of identifying and quantitating drugs in urine, it is an essential quality assurance component that the laboratory monitor and document that all analytical instruments involved in the analysis are maintained and operated properly. The laboratory must establish that the instrument used for a particular analysis is operating adequately and within expected performance specifications. Prior to the start of an assay, the condition of the GC/MS system must be evaluated. The GC/MS operator should check the injection port, detector and oven temperatures, and carrier gas pressure, and perform routine maintenance, as needed, such as clipping of the GC column and replacement of the injector septum and liner. In addition, on a periodic basis, or when a new column is installed, the carrier gas flow rate should also be measured.

Routine maintenance should be performed at least as often as recommended by the manufacturer. Additional maintenance and instrument check schedules should be developed by the laboratory according to its workload and type of assay performed. For example, the laboratory may choose to routinely replenish or replace vacuum pump oil on a quarterly basis. Or, the laboratory may choose to replace injection port liners daily. Other types of maintenance procedures include replacement of filaments, cleaning of the ion source, and replacement of electron multiplier or ion gauge. The specific schedule developed by the laboratory will be dependent upon the type of GC/MS instrument used (e.g., traditional quadrupole, ion-trap), as well as the nature of the extracts analyzed on a particular system. The GC/MS laboratory must maintain records of all routine and nonroutine maintenance performed prior to analysis of specimens, as well as written standard operating procedures for the performance of these tasks [9,26,31,53,80]. Record keeping for maintenance procedures is vitally important to demonstrate the validity of the analysis [31].

B. Instrument Performance Evaluation and Tuning

On a daily basis, it is a good practice to check the pressure in the ion source and in the analyzer, as well as the GC column head pressure to assure that no major system leaks have occurred. For quadrupole operation, a pressure of 10^{-4} torr or better is required so that significant interaction between the ion beam and residual gas molecules does not occur (which causes scattering of the ion beam and loss of sensitivity). For GC/MS, an even lower pressure of 10^{-5} torr is necessary to reduce residual gas in order to prevent significant distortion of mass spectra and reduce background interference. Chemical ionization MS typically requires higher source pressure than EI MS.

Following this initial pressure check, the laboratory should verify that there are no significant air/water leaks in the system by monitoring the intensity of the following ions: m/z 28 (N_2^+), 32 (O_2^+), 40 (Ar^+), and 44 (CO_2^+). If these peaks are abnormally large, an air leak may be indicated. In addition, evaluation of water vapor in the system can be checked by monitoring m/z 18 (H_2O^+) and 19 (H_3O^+). Appropriate instrument maintenance must be conducted before analysis is permitted to proceed.

A GC/MS "tuning procedure" ensures that appropriate mass-to-charge assignments and abundances of specific ions have been established, as well as indicating the need for instrument preventative maintenance. In order to verify proper calibration and operation of the mass spectrometer, the instrument must be tuned daily with an appropriate tuning compound (e.g., perfluorotributyl-

amine, PFTBA) for proper unit resolution and mass assignment. "Resolution" refers to baseline separation between consecutive integral mass peaks. In mass spectrometry, resolution and sensitivity are inversely related. Tuning should be performed at the operating temperature of the ion source.

Autotune procedures typically utilize a preselected set of criteria across a range of mass-to-charge ratios (e.g., m/z 69 to 502 for PFTBA) to optimize source and quadrupole potentials. Alternatively, manual tunes can be used to increase sensitivity over a narrower mass range. Autotune procedures, or optimization of tuning parameters across a wide m/z range using a manual tune, are most useful for analysis of unknown specimens. Narrow-range manual tunes can be useful for some low-level target compound analyses. In this case, either of the abundant ions at m/z 219 or 414 can be used to optimize source potentials, depending on which is closest to the analyte and internal standard ions that will be monitored. Manual tuning is acceptable as long as the MS operator is appropriately trained, the tune procedure is fully documented, and the laboratory's standard operating procedure (SOP) manual describes the task clearly and accurately. It is most common, however, for urine drug-testing laboratories to perform an autotune across the entire mass range (e.g., m/z 69 to 502) at approximately 70 eV [9,26,31,43].

All tune reports should be reviewed thoroughly by the operator before testing is initiated to support compliance with manufacturer and laboratory specifications. Acceptable limits should be established for the ion focus, ion appearance and peak width, abundance of selected ions, isotope ratios, and mass slope, as appropriate for the GC/MS instrument. Critical tune values should be monitored on a regular basis, and all tune reports, including unacceptable ones, should be archived as important supporting forensic documents. Autotune, or manual tune, information should be available during review of the batch to ensure that instruments were performing as expected prior to analysis of specimens. These records may be filed with the pertinent batch, or filed in a manner to permit easy retrieval [72].

C. Chromatographic Performance

The chromatographic performance of an assay should also be assessed before the analysis of specimens. This is achieved readily by the injection of an unextracted performance standard including analyte(s) and internal standard(s). Use of an unextracted standard removes sources of variation due to the extraction procedure and matrix interferences. In addition to permitting evaluation of the quality of the chromatographic system, the analysis

of an unextracted standard serves to verify that the analyte(s) of interest elute at the expected retention time, that all MS acquisition windows are appropriately set, and that no unexpected adsorptive system losses have occurred. Evaluation criteria in the SOP manual should include a thorough description of peak shape, resolution, and signal abundance requirements.

One approach to evaluation of acceptable chromatography for a single specimen and/or an entire batch of specimens may be defined and assessed by the following criteria:

1. The analyte of interest is present at the correct retention time.
2. The peak of interest obtained from the total ion chromatogram and each individual ion chromatogram is inspected visually for geometric symmetry. A gaussian peak shape (see Figure 1) is required and shall have no greater than 50% tailing using the following procedure:
 - a. Draw a vertical line from the apex through the center of the peak to the baseline.
 - b. Draw a line parallel to the baseline at 10% of the peak height.
 - c. Measure the distance "a" from the leading edge to the centerline.
 - d. Measure the distance "b" from the centerline to the trailing edge.
 - e. Calculate the ratio of distance "a" and distance "b".
 - f. If the ratio is greater than 2 or less than 0.5, performance is unacceptable.
3. The peak of interest is inspected visually for the presence of unresolved peaks. The maximum allowable valley between two adjacent peaks must not exceed 10% of the analyte peak height.
4. Abundance or signal-to-noise levels of the internal standard ions must meet established minimum criteria.

Failure to meet any of these criteria may provide cause for reinjection or reextraction of a specimen. A complete review of methods for evaluation of chromatographic performance is beyond the scope of this paper; however, acceptable alternative criteria for evaluation of chromatographic acceptability can be found in many textbooks and other reference materials [30,36,49,51,60, 63]. Some instrument software designers are also developing programs for automated evaluation of chromatography.

In addition to daily chromatographic checks prior to batch analysis, performance of the chromatographic column should be evaluated upon installation and periodically thereafter. This can be accomplished by the analysis of a variety of "test mixtures," such as a Grob's Test Mix

[30], to assess column performance. These mixtures generally contain several compounds (e.g., early and late eluters) which are selected to evaluate certain aspects of chromatographic performance, including peak shape and sensitivity. From a practical perspective, the forensic urine drug-testing laboratory may also choose to evaluate acceptable chromatographic performance for selected drug analytes (e.g., amphetamine, phencyclidine, 6-acetylmorphine, codeine) after column installation. Deterioration in performance can then be followed by comparison of the initial chromatogram with subsequent chromatographic data.

III. ASSAY CALIBRATION

The term "*assay calibration*" refers to the process of developing a mathematical model that attempts to predict the value of an independent variable (e.g., concentration) based on the value of the dependent variable (e.g., peak height, peak area ratios). Calibration has sometimes been referred to as inverse prediction or discrimination. This is in contrast to the term "regression," which was described earlier in this paper with respect to linearity of the analytical method. *Regression* refers to a functional relationship between two or more correlated variables [6,21]. In forensic urine drug-testing laboratories, assay calibration is necessary to determine whether an analyte is present in an unknown sample at or above a pre-established administrative cutoff value, as well as to determine the accurate quantitative concentration of the analyte under certain circumstances [72]. Currently, it is most common to calibrate the assay for quantitative measurement using a single, abundant ion fragment that is characteristic of the analyte of interest. Additional ions, referred to as qualifying ions, are used to support the qualitative identification of the analyte in the unknown sample.

Three approaches to assay calibration are commonly utilized in forensic urine drug-testing laboratories: multi-point calibration curves, single-point calibration curves,

and historical calibration curves. The first and probably the most widely employed method of calibration is the preparation of a *multi-point calibration curve*. Experimentally derived multi-point calibration curves are used to cover the concentration range of the samples to be measured, thereby improving the confidence limits associated with the calibration itself. Calibration samples (standards) are prepared from mixtures of different amounts of known concentration of analyte with a fixed amount of internal standard. Specific ion-abundances are measured for the analyte and internal standard, a ratio calculated, and a calibration curve generated using a simple least squares regression model, for example. If the laboratory elects to use a multi-point calibration curve, it must include calibrators that bracket the cutoff concentration. Although many laboratories include a calibrator at the cutoff concentration, it is not required [72].

The calculated concentration of all standards utilized to construct the calibration curve should be within $\pm 20\%$ of their respective target concentrations. If more than three standards are utilized to construct a calibration curve, it is permissible to delete one calibrator for cause (e.g., poor recovery) provided it is not a calibrator at the cutoff concentration. It is not acceptable to eliminate a calibration point solely in order to bring the quality control results within range, although there is some disagreement on this issue since one might expect that the probability of detecting/obtaining an "outlier" will increase as the number of calibrators analyzed in a batch increases [15,72].

Evaluation criteria for linearity of the multi-point calibration curve include the correlation coefficient (r), coefficient of determination (R^2), the slope (m), y-intercept (b), and quantitative value of each data point. The *correlation coefficient* is a measure of the intensity of association between two variables, X and Y . In calibration, the correlation coefficient ranges from 0 to 1; 0 indicates no correlation and 1 indicates a perfect correlation. A correlation coefficient may indicate a high degree of relation between variables, but the generated model (regression equation) may give an inadequate fit for the data. In contrast, the *coefficient of determination* is a measure of "goodness of fit" and is the proportion of variation in the data explained by the regression model. It also ranges from 0 to 1, with 0 indicating a complete lack of fit and 1 indicating a perfect fit. Generally accepted criteria for acceptable linearity using deuterated internal standards for calibration include a correlation coefficient and/or coefficient of determination of at least 0.990, and a y-intercept close to zero, although slightly positive or negative y-intercept values are also acceptable. Because of the potential for negative and positive bias, y-intercept

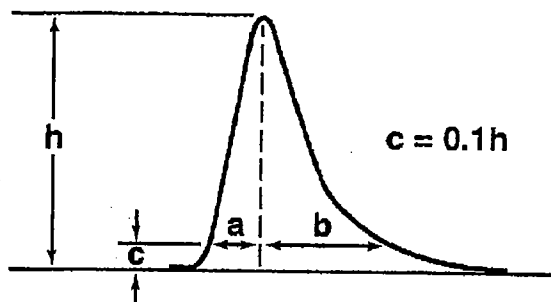


Figure 1. Theoretical chromatographic peak.

values should be monitored on a regular basis and tracked for development of trends.

For assay calibration, the laboratory must also establish whether the regression line will be forced through the origin (a "no-intercept" model) or will not be forced through the origin (an "intercept" model). In the intercept model, R^2 is the proportion of variance explained by the model. In contrast, R^2 produced by the no-intercept model is a measure of the degree of dispersion around zero and describes the proportion of variance around zero explained by the model [7]. Most GC/MS software programs allow for the user to select between these two approaches. The selection of the appropriate model to be used for a particular assay will depend on several factors, including the type and sensitivity of instrument, the analytical method, the method of ionization, the required analyte detection, and reporting limits. As a general rule, it has been suggested that one should assume that an intercept model is correct until proven otherwise via statistical testing of the intercept [6-8,14]. If statistical testing of the intercept indicates that it is not different from zero (e.g., $b = 0$), then the no-intercept model is appropriate and should be used. However, selection of a no-intercept model implies that the assay limit of detection is zero, which is not the case for GC/MS urine drug-testing assays due to instrument and matrix background effects. Therefore, in most instances regression through the origin should not be used in assay calibration.

The second most commonly employed approach to assay calibration is a *single-point calibration*. In this case, a single standard containing analyte at the assay cutoff concentration is used to establish the cutoff concentration in order to determine whether a specimen is positive or negative. Laboratories then include quality control materials at concentrations below, at, and above the cutoff concentration to demonstrate linearity. The quantitative results for the quality control samples used in single-point calibration must fall within $\pm 20\%$ of their respective target concentrations.

The third approach to assay calibration uses construction of a *historical multi-point calibration curve*. The laboratory establishes the calibration (as above) and verifies that the calibration has not changed between batches via analysis of control samples, one of which is at the cutoff concentration. Use of historical calibration curves is acceptable only if the laboratory has demonstrated linearity and precision of the calibration curve over time. If shifts in response ratios are observed or new internal standard materials are employed, a new standard curve must be prepared. In addition, all batches must include at a minimum a blank and two controls, one at or near the cutoff.

During assay calibration, the laboratory also establishes acceptance ranges for retention time and ion ratios. These acceptance ranges are then used to evaluate each calibrator, control, and unknown specimen in the batch (see Section V). For single-point calibration assays, the acceptable limits are determined from the calibrator at the cutoff. For multi-point or historical calibration curves, acceptable limits may be determined from either the calibrator at the cutoff or from the average of all the calibrators analyzed. If historical calibration curves are used, verification of correct retention time and ion ratios is performed by examination of the "at or near cutoff" quality control sample. In this case, the retention time and ion ratios of this sample must fall within the ranges established by the historical curve, or the assay must be recalibrated [72]. Any of the approaches for determining retention time and ion ratio criteria discussed above are considered acceptable; however, the laboratory must apply the acceptable ranges consistently to all calibrators, controls, and specimens in the batch.

IV. QUALITY CONTROL

A. Required Quality Control Samples

Each assay batch must include a minimum total of 10% open and blind positive and negative quality control samples in an appropriate urine matrix [70]. External blind quality control samples are not required, but are highly recommended. Quality control samples may be purchased commercially or prepared from a different source or lot of standard material other than that used to prepare calibrators. The use of different sources and lot numbers is recommended in order to eliminate systematic quantitative bias in the GC/MS assay that may otherwise go undetected. However, at a minimum, quality control materials should be prepared using standard material prepared from a different weighing or vial of source material other than that used to prepare the calibrators. The target concentration of at least one control must be within approximately 125% of the cutoff concentration; other controls should be prepared at appropriate concentrations in order to regularly assess accuracy below and/or above the assay cutoff concentration. It is recommended that a highly concentrated control sample be diluted in a similar manner as diluted specimens during aliquoting in order to verify the accuracy of the dilution technique, if one is routinely used to prepare presumptive positive specimens for confirmation [72].

In order to evaluate the efficiency of the hydrolysis process, cannabinoid and opiate control samples containing conjugated 11-nor- Δ^9 -tetrahydrocannabinol-9-car-

boxylic acid and morphine, respectively, should be assayed. Negative urine samples spiked with these reference materials can be prepared, or as an alternative, hydrolysis control samples can be prepared from a combined urine pool of previously confirmed specimens. (It is not acceptable to use regulated specimens for this or any other purpose until after completion of the required storage time.)

Finally, it is highly recommended, but not required, that the laboratory use quality control materials that include the addition of potentially interfering substances in its GC/MS assays. These might include compounds that are structurally related to the analyte of interest, or closely eluting compounds. For example, the laboratory may choose to include a control containing hydrocodone, hydromorphone, or oxycodone in its opiate assay to demonstrate that codeine and morphine are correctly identified and accurately quantitated in the presence of potential interferents. Another example is that of the inclusion of a control containing phenylpropanolamine, ephedrine, phentermine, and/or pseudoephedrine in GC/MS assays that utilize a periodate procedure.

B. Verification of Quality Control Materials

Prior to the use of reference materials to prepare calibrator, control, or internal standard material in the laboratory, the laboratory is required to verify, *independently* from the supplier, that its chemical identity is correct and that it is of acceptable purity and concentration. The laboratory may perform this verification itself, or may refer it to another laboratory. At a minimum, most laboratories perform a full-scan GC/MS analysis to verify the chemical identity and purity of the material and compare the obtained spectra with that of available library spectra to determine that significant impurities are not present in the material which might interfere with the method. The appropriate derivatization procedure for the analyte of interest is employed, as well as the GC conditions routinely used for the assay.

The isotopic purity of the internal standard can also be verified with the same procedure described above. In addition to full-scan analysis, the laboratory may also evaluate the deuterated internal standard in selected-ion monitoring (SIM) mode prior to use.

Additional methods for verification of chemical identity and purity may involve measurement of physical constants, such as melting point or refractive index, as well as use of other analytical techniques (HPLC, IR, NMR, TLC, or UV/VIS) to detect nonpolar or nonvolatile impurities [63]. Verification of concentration is most often evaluated indirectly by preparation of calibrators or

controls at known concentrations and analysis in routine batches.

The laboratory must establish specific evaluation criteria for reference materials, such as spectral match requirements, percent isotopic purity required, and quantitative results. The laboratory must retain documentation of all verification procedures performed [72]. The laboratory may then use the reference material to prepare calibrators for controls for routine use. Of course, these new calibrators and controls must then be themselves validated for concentration (e.g., $\pm 20\%$ of the target concentration) prior to routine use.

C. Evaluation of Quality Control Results

There are two major approaches to evaluation of quality control results applied to urine drug testing. The first approach is the use of a fixed-criterion quantitative acceptance range. In this case, the measured concentration of control samples must be within $\pm 20\%$ of the target concentration. A more detailed discussion of the rationale underlying this criterion has already been discussed in Section I-B.

The second approach is to use modifications of Westgard Quality Control Rules to evaluate results. In this approach, the laboratory establishes warning limits and out-of-control limits for the assay based upon the validated mean and standard deviation for the control sample. A thorough description of Westgard rules may be found in several sources [15,35,36,75-77]. Westgard rules are usually not directly applicable to GC/MS forensic testing due to the limited number of quality control data points obtained in a batch, the large number of independent variables associated with GC/MS systems, and the acceptance of potentially out-of-range data. However, if *coupled* with a fixed quantitative accuracy requirement of $\pm 20\%$ for control materials, the rules can be extremely useful for the purposes of evaluating the GC/MS assay for development of trends and systematic biases [38]. Also, other approaches to the evaluation of quality control data, including an ANOVA approach, have been described [37,42].

The laboratory's SOP manual must thoroughly describe the quality control evaluation criteria to be used and must include a policy for the required course of corrective action if quality control sample results fail to meet acceptance criteria. In order to assess laboratory performance, all control data, including out-of-limit data, should be recorded in the quality control log in Levey-Jennings or Shewart chart format [15,27,62,72,75-77]. Out-of-limit data should include documentation of required corrective action. It may be acceptable to reinject a quality control

sample one additional time. If the results are still unacceptable, other minimally acceptable protocols include:

1. Reinjection of calibrators, followed by reprocessing of *all* quality control samples and routine specimen data against the new calibration, *if* the time since the last injection is not excessive and the instrument has not been retuned;
2. Reinjection of all calibrators, quality control samples, and routine specimens; and
3. Acceptance of negative test results that are less than the LOD and reextraction of all other specimens in the batch.

V. CRITERIA FOR DESIGNATING A POSITIVE TEST RESULT

A. Chromatographic Criteria

Criteria for designating a positive test result include chromatographic and spectral identification. Chromatographic identification of an analyte requires comparison of the retention time of the specimen with that of a calibrator at the cutoff or the average of multiple calibrators (either approach is considered acceptable). Generally, the retention time of the analyte should be within $\pm 2\%$ of the retention time as established by the calibrator(s) [11-13,72].

B. Ion Ratios

Further, identification of an analyte requires comparison of ion ratios or full spectral data of the unknown with preestablished ion ratios or full-scan mass spectra, respectively. Acceptable ion ratios for the analyte and its corresponding internal standard are usually calculated using ion abundance data obtained for the standard prepared at the cutoff concentration or by determining the mean ion ratios for all calibrators. It has been demonstrated statistically that while full-scan mass spectrometric data provide the maximum confidence for analyte identification, a minimum of three structurally significant ions generated under electron ionization conditions appear to provide adequate information for an identification [12,64]. If the regulated urine drug-testing laboratory uses EI, it is currently required to use a minimum of two ion ratios for identification of the analyte and at least one ion ratio for the internal standard [72]. Of course, CI-generated spectra do not always meet the three-ion criteria because less extensive fragmentation is generally observed with this technique. However, this limitation is offset by the production of high m/z ions characteristic of the analyte and increased specificity with selection of appropriate reagent gases [11,12,26,81].

The ion ratios for the analyte and its corresponding internal standard obtained for the unknown should not differ by more than $\pm 20\%$ of the target ion ratio and acceptance criteria must be uniformly applied to all specimens within the batch [72]. The establishment of this 20% acceptance criteria for ion abundance ratios has been determined to be appropriate based on ion statistics [11-13,64]. Different ion ratio criteria cannot be applied to different specimens, calibrators, or controls within the batch.

C. Mass Spectral Match

To ensure adequate mass spectral match quality for laboratories utilizing full-scan acquisition, unknown mass spectra must be compared with reference spectra, and fit or match quality values must be computed. The laboratory should determine allowable limits of acceptability in accordance with laboratory studies and manufacturer's recommendations (e.g., 950 or greater out of a scale of 1,000). It is known that reference spectra in spectral libraries may at times differ from that obtained from analysis of actual specimens. This may be due to the type of instrument used, the particular algorithms used to generate a match, the number of ions used to establish a match, type of reagent gas, electron energy, or type of derivatization employed, among other factors. Therefore, the laboratory may consider establishing its own spectral library for analytes of interest, providing that manufacturer's specifications continue to be met and that generated spectra do not differ *significantly* from published reference sources.

In addition to spectral match requirements, the signal-to-noise ratio at the apex of the integrated peak of the analyte and its internal standard should be equal to or greater than a minimum of 10:1 at the assay's LOQ.

D. Quantitative Result

Regardless of the detection technique, in order to be designated as positive, the measured concentration of a specimen must be equal to or exceed the established assay cutoff concentration. Quantitative results around the cutoff must be truncated, rather than rounded up to the nearest whole number, so that the statistical bias is toward a "negative" result.

Specimens directed for GC/MS retest analyses are not subject to cutoff concentrations and are reported as reconfirmed if the concentration is equal to or greater than the LOD of the method. The specimen retest must also meet all other criteria for designating a positive test result. Quantitative reports should be provided only upon written request of the Medical Review Officer.

If the specimen concentration exceeds the linearity limits of the assay and the specimen was not diluted accordingly, the report must state: "the concentration of 'analyte' is greater than 'the established linearity limit'." Also, all criteria for designating a positive test result must be satisfied including chromatographic performance, ion ratios, and retention time data.

VI. MISCELLANEOUS FACTORS

A. Data Presentation

Although presentation of GC/MS data is highly variable among laboratories, printouts for all specimens (calibrators, controls, and positive and negative specimens) must include specimen identification information, total ion chromatogram illustrating entire acquisition window, individual selected ion chromatograms drawn to an approximate 0.5-minute window, mass spectrum (if applicable), spectral data including abundance and ion ratios or spectral match, retention time data, and concentration of analyte. In addition, data file name, date and time of injection, and MS operator name are highly desirable. Figures 2 and 3 illustrate typical GC/MS reports for confirmation of benzoylecgonine in urine using the Hewlett-Packard Mass Selective Detector and the Finnigan-MAT Ion Trap Mass Spectrometer, respectively. Further, a summary sheet presented in tabular format including injection sequence and specimen identification information, spectral data, and quantitative results should be prepared for ease of batch review (see Figure 4).

B. Dilution Protocols

Dilution protocols may be developed based upon the relationship of the immunoassay response and the quantitative result. Specimens may be diluted with GC/MS verified negative urine (or possibly purified water) prior to GC/MS confirmation in order to avoid carryover, prevent chromatographic overload, and to obtain acceptable chromatographic results. It is recommended that a quality control sample be diluted in the same manner as any routine specimen to assess the accuracy of the dilution.

A retest specimen may be tested by an immunoassay procedure in order to determine the need for dilution prior to GC/MS analysis. If dilution protocols are routinely applied by the laboratory, these procedures should be clearly described in the laboratory's SOP manual [72].

C. Reinjection of Extracts

Reinjection of specimen extracts may be necessary due to a failed injection or chromatographic overload. The laboratory must stipulate the number of acceptable reinjections (in regulated laboratories, more than one reinjection is generally considered unacceptable) and the maximum time after the end of the batch that reinjection is acceptable. If the reinjection is necessary due to column overload, the laboratory may elect to establish a policy whereby additional reconstitution solvent is added to the extraction vial prior to reinjection. However, such handling must be clearly recorded on laboratory documents, and the laboratory must establish minimum criteria for internal standard signal-to-noise and ion abundance to ensure that results continue to be reliable.

It is unacceptable to continue to reinject or reextract a specimen in order to "force" a negative or positive result. If a specimen fails acceptance criteria it can be reinjected one time and scheduled for re-extraction one time. However, if it continues to fail to meet criteria for the same reason, the specimen must be reported as negative. When reinjections are performed, it is necessary to reinject at least one standard and/or control in order to verify assay performance at the time of the reinjection. All initial and reinjection data, including failed or unacceptable data, must be maintained. Finally, retuning prior to reinjection is not permitted [72].

VII. REVIEW AND EVALUATION OF GC/MS DATA

All data must be thoroughly reviewed by a minimum of two individuals to verify compliance with the methods specified in the laboratory's procedure manual and to identify clerical and/or analytical errors. Batch acceptance criteria include within-range standards and controls and acceptable MS tune, chromatographic performance, ion abundance (adequate signal-to-noise ratio), and ion ratios or mass spectral match quality. Also, the laboratory may choose to monitor the consistency (reproducibility) of the ion abundance for the internal standard to ensure that it has been added appropriately, and at the correct concentration, to each calibrator, control, and unknown in confirmatory analyses. Furthermore, an acceptable calibration curve must be obtained, a lack of carryover must be demonstrated, and chain-of-custody documentation must be in order.

The SOP must also address the handling and reporting of results when duplicate extracts are assayed, or diluted and undiluted extracts are analyzed. Acceptance criteria for duplicates must specify minimum correlation of quan-

74

QUANTITATION REPORT FOR BE ON : 5972 - MS

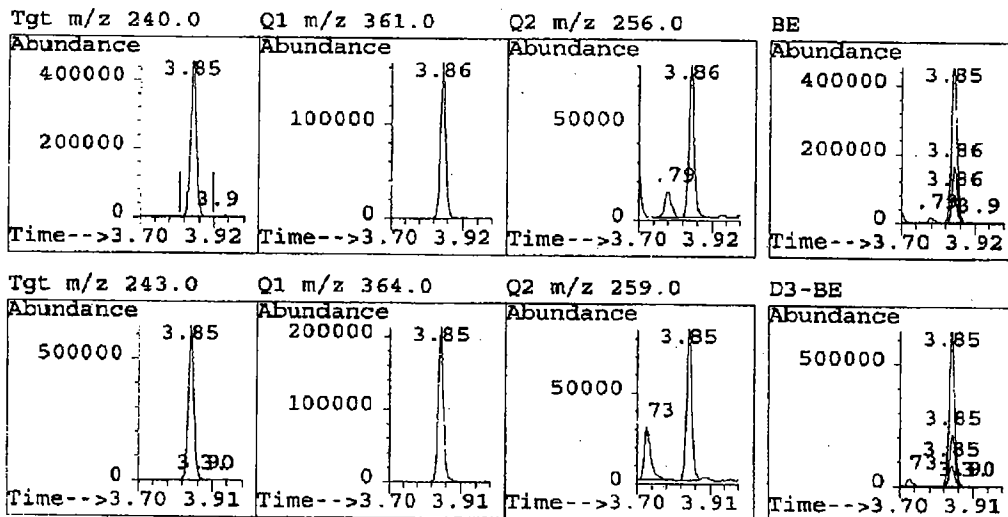
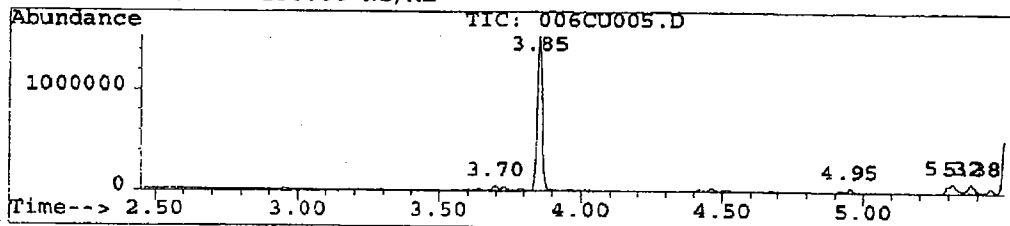
Data File : C:\HPCHEM\1\DATA\C5AUG96A.10A\006CU005.D
 Tune File Name : C:\HPCHEM\1\5972\ATUNE.U
 Tune Date : 5 Aug 96 9:42 am
 Acq Method Name : BEDRL.M Calib date : 05 Aug 96 1:24 pm
 Sample Name/Barcode : 150 NG/ML
 Acq date/Exp.Barcode : 5 Aug 96 11:43 am /

Retention Time 3.85 BE +/- 1.00% = 3.81 - 3.89 min
 Retention Time 3.85 D3-BE +/- 1.00% = 3.81 - 3.89 min
 R.R.T. = 1.001 Unknown target ion / ISTD target ion = 0.75

BE => 240.0 = 528009 361.0 = 183619 256.0 = 89775
 D3-BE => 243.0 = 707972 364.0 = 244509 259.0 = 95708

BE => 361.0/240.0 = 34.8 +/- 20.0% rel = 27.8 - 41.8
 BE => 256.0/240.0 = 17.0 +/- 20.0% rel = 13.6 - 20.4
 D3-BE => 364.0/243.0 = 34.5 +/- 20.0% rel = 27.6 - 41.4
 D3-BE => 259.0/243.0 = 13.5 +/- 20.0% rel = 10.8 - 16.2

Concentration = 144.84 ** CUTOFF CALIBRATOR **
 Cutoff limit = 150.00 NG/ML



BE : RT extraction window from 3.70 to 4.00 min
 D3-BE : RT extraction window from 3.70 to 4.00 min

Page 11

Printed on : 05 Aug 96 1:24 pm

Figure 2. GC/MS report for confirmation of benzoylecgonine in urine using the Hewlett-Packard Mass Selective Detector.

75

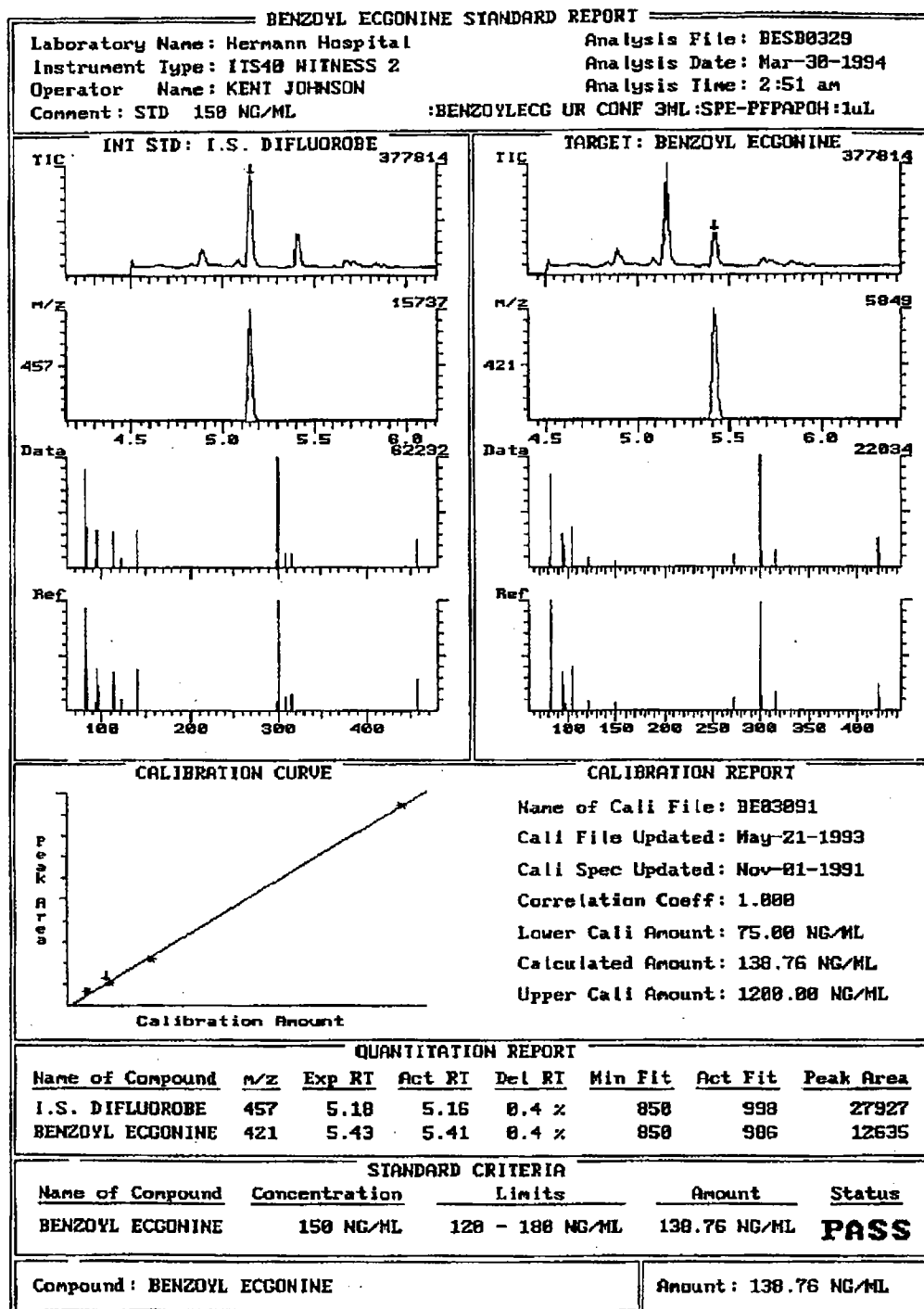


Figure 3. GC/MS report for confirmation of benzoylecgonine in urine using the Finnigan-MAT Ion Trap Mass Spectrometer. (Courtesy of Kent Johnson of Hermann Hospital: Houston, TX.)

Batch Summary Report **TOXICOLOGY LAB**

Batch Dir.: 05AUG96A.10A

Acquisition Date: 8/5/96 10:48 AM

Instrument Name: 5972 - MSD2

Drug Class: BENZOYLECGONINE

Operator: B. GOLDBERGER

Batch Name: 05AUG96A.10A

Original Method File: C:\HPCHEM\1\METHODS\BEDRL.M

Ion Ratio Range: +/-20%

Data Path: C:\HPCHEM\1\DATA\05AUG96A.10A

Retention Time Range: +/-1%

| Cutoff File Information | | | | | Quantitation Database | | | | | |
|-------------------------|---------------|--------------|------------|-------------|-----------------------|-----------|---------------|---------------|--------------|--------------|
| Compound Name | | Cutoff Conc. | File Name | Calc. Conc. | Sample Name | RT | Ratio 1 | Ratio 2 | ISTD Ratio 1 | ISTD Ratio 2 |
| BE | | 150 | 006CU005.D | 144.84 | 150 NG/ML | 3.85 | 34.78 | 16.98 | 34.54 | 13.52 |
| Run No. | Compound Name | Result | File Name | Calc. Conc. | Sample Name | Target RT | Target Ratio1 | Target Ratio2 | ISTD Ratio1 | ISTD Ratio2 |
| 1 | BE | POSITIVE | 001SP001.D | 155.98 | UNEXTRACTED STD | 3.85 | 34.94 | 16.80 | 33.97 | 13.24 |
| 2 | BE | Blank | 002BL000.D | 0.00 | SOLVENT BLANK | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 3 | BE | No ISTD | 003NE002.D | 0.00 | BLANK | 3.85 | 0.00 | 0.00 | 0.00 | 0.00 |
| 4 | BE | Good Neg. | 004NE003.D | 0.00 | BLANK + IS | 3.85 | 0.00 | 0.00 | 33.45 | 13.41 |
| 5 | BE | Calibrator | 005CA004.D | 74.45 | 75 NG/ML STD | 3.85 | 33.89 | 16.59 | 33.62 | 13.39 |
| 6 | BE | Cutoff | 006CU005.D | 144.84 | 150 NG/ML STD | 3.85 | 34.78 | 16.98 | 34.54 | 13.52 |
| 7 | BE | Calibrator | 007CA006.D | 1000.12 | 1000 NG/ML STD | 3.85 | 35.43 | 17.03 | 34.87 | 13.65 |
| 8 | BE | Blank | 008BL000.D | 0.00 | SOLVENT BLANK | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9 | BE | POSITIVE | 009SP007.D | 270.22 | R98-00001*5 | 3.86 | 35.82 | 17.05 | 35.34 | 13.49 |
| 10 | BE | Blank | 010BL000.D | 0.00 | SOLVENT BLANK | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 11 | BE | Negative | 011SP008.D | 135.59 | R98-00002*5 | 3.86 | 35.25 | 16.91 | 35.34 | 13.73 |
| 12 | BE | Blank | 012BL000.D | 0.00 | SOLVENT BLANK | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 13 | BE | Negative | 013SP009.D | 0.00 | QC NEGATIVE | 3.85 | 0.00 | 0.00 | 35.81 | 13.60 |
| 14 | BE | POSITIVE | 014SP010.D | 188.58 | QC POSITIVE | 3.85 | 36.11 | 17.40 | 34.66 | 13.98 |

End of Batch

Figure 4. GC/MS Batch Summary Review form.

titative results, usually $\pm 20\%$ if both results fall within the assay's linear range. In addition, both of the results must be equal to or greater than the mandated cutoff concentration, although use of the lowest value is recommended. The averaged results or a single value, diluted or undiluted, may be reported if both results meet acceptance criteria, and SOP requirements are consistently applied.

Review of specimen data should include comparison of the initial immunoassay response with the GC/MS result, an evaluation of chromatographic performance including presence of interfering or extraneous peaks, retention time, minimum ion abundance, ion ratios or mass spectral match quality, quantitation, extraction efficiency, potential for carryover, and chain-of-custody documentation.

REFERENCES

1. Armbruster DA, Tillman MD, Hubbs LM: Limit of detection (LOD)/Limit of quantitation (LOQ): Comparison of the empirical and the statistical methods exemplified with GC/MS assays of abused drugs; *Clin Chem* 40:1233; 1994.
2. Arnoux P, Morrison R: Drug analysis of biological samples. A survey of validation approaches in chromatography in the United Kingdom pharmaceutical industry; *Xenobiotica* 22:757; 1992.
3. Blanke RV: *Validation of the purity of standards*. Abbott Laboratories (Diagnostic Division): Abbott Park, IL; 1989.
4. Blau K, Halket JM (Eds): *Handbook of Derivatives for Chromatography*, 2nd ed; John Wiley & Sons: New York, NY; 1993.
5. Blau K, King GS (Eds): *Handbook of Derivatives for Chromatography*; Heyden & Son: London, U.K.; 1978.
6. Bonate PL: Concepts in calibration theory. Part I: Regression; *LC-GC* 10:310; 1992.
7. Bonate PL: Concepts in calibration theory. Part II: Regression through the origin — When should it be used? *LC-GC* 10:378; 1992.

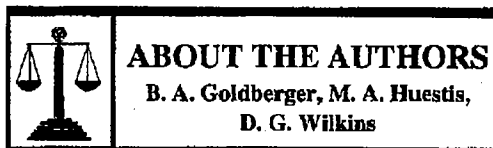
8. Bonate PL: Concepts in calibration theory. Part III: Weighted least-squares regression; *LC-GC* 10:448; 1992.
9. Boyd RK, Henion JD, Alexander M, Budde WL, Gilbert JD, Musser SM, Palmer C, Zurek EK: Mass spectrometry and good laboratory practices; *J Am Soc Mass Spectrom* 7:211; 1996.
10. Buick AR, Doig MV, Seal SC, Land GS, McDowal RD: Method validation in the bioanalytical laboratory; *J Pharm Biomed Anal* 8:629; 1990.
11. Baldwin R, Bethem RA, Boyd RK, Budde WL, Cairns T, Gibbons RD, Henion JD, Kaiser MA, Lewis DL, Matusik JE, Sphon JA, Stephany RW, Trubey RK: Report — 1996 ASMS Fall Workshop: Limits to confirmation, quantitation, and detection; *J Am Soc Mass Spectrom* 8:1180; 1997.
12. Cairns T, Siegmund EG, Stamp JJ: Evolving criteria for confirmation of trace level residues in food and drugs by mass spectrometry. Part I; *Mass Spectrom Rev* 8:93; 1989.
13. Cairns T, Siegmund EG, Stamp JJ: Evolving criteria for confirmation of trace level residues in food and drugs by mass spectrometry. Part II; *Mass Spectrom Rev* 8:127; 1989.
14. Cassidy R, Janoski M: Is your calibration curve linear? *LC-GC* 10:692; 1992.
15. Cembrowski GS, Carey RN: *Laboratory Quality Management*; ASCP Press: Chicago, IL; 1989.
16. Claeys M, Markey SP, Maenhaut W: Variance analysis of error in selected ion monitoring assays using various internal standards. A practical study case; *Biomed Mass Spectrom* 4:122; 1977.
17. Craft NE, Byrd GD, Hilpert LR: Preparation and certification of standard reference material 1507: 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid in freeze-dried urine; *Anal Chem* 61:540; 1989.
18. Dadgar D, Burnett PE, Choe MG, Gallicano K, Hooper JW: Application issues in bioanalytical method validation, sample analysis and data reporting; *J Pharm Biomed Anal* 13:89; 1995.
19. Davidian M, Carroll RJ, Smith W: Variance functions and the minimum detectable concentration in assays; *Biometrika* 75:549; 1988.
20. Dorschel CA, Ekmanis JL, Oberholtzer JE, Warren FV, Bidlingmeyer BA: LC detectors — Evaluation and practical implications of linearity; *Anal Chem* 61:951A; 1989.
21. Edwards AL: *An Introduction to Linear Regression and Correlation*, 2nd ed; WH Freeman and Company: New York, NY; 1976.
22. Ellerbe P, Long T, Welch MJ: The determination of amphetamine and methamphetamine in a lyophilized human urine reference material; *J Anal Toxicol* 17:165; 1993.
23. Ellerbe P, Tai SS-C, Christensen RG, Espinosa-Leniz R, Paule RC, Sander LC, Sniegoski LT, Welch MJ, White E: The certification of cocaine and benzoylecgonine in a human urine standard reference material; *J Anal Toxicol* 16:158; 1992.
24. ElSohly MA, Stanford DF, Sherman D, Shah H, Bernot D, Turner CE: A procedure for eliminating interferences from ephedrine and related compounds in the GC/MS analysis of amphetamine and methamphetamine; *J Anal Toxicol* 16:109; 1992.
25. Fenton J, Mummert J, Childers M: Hydromorphone and hydrocodone interference in GC/MS assays for codeine and morphine; *J Anal Toxicol* 18:159; 1994.
26. Foltz RL, Fentiman AF, Foltz RB: *GC/MS Analysis for Abused Drugs in Body Fluids* — NIDA Research Monograph 32; National Institute on Drug Abuse: Rockville, MD; 1980.
27. Garfield FM: *Quality Assurance Principles for Analytical Laboratories*; AOAC International: Arlington, VA; 1991.
28. Garland WA, Barbalas MP: Applications to analytic chemistry: An evaluation of stable isotopes in mass spectral drug assays; *J Clin Pharmacol* 26:412; 1986.
29. Green JM: A practical guide to analytical method validation; *Analyt Chem News Features* p 305A; May 1, 1996.
30. Grob K, Grob G, Grob K Jr: Testing capillary gas chromatographic columns; *J Chromatogr* 219:13; 1981.
31. Henion J, Covey T, Maylin G: Forensic mass spectrometry in equine drug testing; *Spectra* 10:21; 1985.
32. Hornbeck CL, Carrig JE, Czarny RJ: Detection of a GC/MS artifact peak as methamphetamine; *J Anal Toxicol* 17:257; 1993.
33. Inman EL, Frishmann JK, Jimenez PJ, Winkel GD, Persinger ML, Rutherford BS: General method validation guidelines for pharmaceutical samples; *J Chromatogr Sci* 25:252; 1987.
34. Joern WA: GC/MS assay of the marijuana carboxy metabolite: Urine interference with the dimethyl derivative; *J Anal Toxicol* 16:207; 1992.
35. Kaplan LA, Pesce AJ: *Clinical Chemistry: Theory, Analysis, and Correlation*; CV Mosby: St. Louis, MO; 1984.
36. Kaplan A, Szabo L: *Clinical Chemistry: Interpretation and Techniques*, 2nd ed; Lea & Febiger: Philadelphia, PA; 1983.
37. Karnes HT, March C: Precision, accuracy, and data acceptance criteria in biopharmaceutical analysis; *Pharmacol Res* 10:1420; 1993.
38. Karnes HT, Shiu G, Shah VP: Validation of bioanalytical methods; *Pharmacol Res* 8:421; 1991.
39. Knapp DR: Chemical derivatization for mass spectrometry; *Methods Enzymol* 193:314; 1990.
40. Knapp DR: *Handbook of Analytical Derivatization Reactions*; John Wiley & Sons: New York, NY; 1979.
41. Lang JR, Bolton S: A comprehensive method validation strategy for bioanalytical applications in the pharmaceutical industry. 1. Experimental considerations; *J Pharm Biomed Anal* 9:357; 1991.
42. Lang JR, Bolton S: A comprehensive method validation strategy for bioanalytical applications in the pharmaceutical industry. 2. Statistical analyses; *J Pharm Biomed Anal* 9:435; 1991.
43. Lawson AM, Gaskell SJ, Hjelm M: Methodological aspects on quantitative mass spectrometry used for accuracy control in clinical chemistry; *J Clin Chem Clin Biochem* 23:433; 1985.
44. Lawson GM: Defining the limit of detection and limit of quantitation as applied to drug abuse testing: Striving for a consensus; *Clin Chem* 40:1218; 1994.
45. Liu RH, Baugh DL, Allen EE, Salud SC, Fentress JG, Chadha H, Walia AS: Isotopic analogue as the international standard for quantitative determination of benzoylecgonine — Concerns with isotopic purity and concentration level; *J Forensic Sci* 34:986; 1989.
46. Liu RH, Foster G, Cone EJ, Kumar SD: Selecting an appropriate isotopic internal standard for gas chromatography/mass spectrometry analysis of drugs of abuse —

- Pentobarbital example; *J Forensic Sci* 40:983; 1995.
47. Liu RH, McKeehan AM, Edwards C, Foster G, Bensley WD, Langner JG, Wallia AS: Improved gas chromatography/mass spectrometry analysis of barbiturates in urine using centrifuge-based solid-phase extraction, methylation with d_5 -pentobarbital as internal standard; *J Forensic Sci* 39:1504; 1994.
 48. Long GL, Winefordner JD: Limit of detection; *Anal Chem* 55:712A; 1983.
 49. McNair HM: Method development in gas chromatography; *LC-GC* 11:794; 1993.
 50. Millard BJ: *Quantitative Mass Spectrometry*; Heyden & Sons: London, U.K.; 1978.
 51. Moffat AC, Jackson JV, Moss MS, Widdop B (Eds): *Clarke's Isolation and Identification of Drugs*, 2nd ed; The Pharmaceutical Press: London, U.K.; 1986.
 52. Moore JM: The application of chemical derivatization in forensic drug chemistry for GC and HPLC methods of analysis; *Forensic Sci Rev* 2:80; 1990.
 53. Nadkarni RA: The quest for quality in the laboratory; *Anal Chem* 63:675A; 1991.
 54. Needleman SB, Romberg RW: Limits of linearity and detection for some drugs of abuse; *J Anal Toxicol* 14:34; 1990.
 55. Nix ABJ, Wilson DW: Assay detection limits: Concept, definition, and estimation; *Eur J Clin Pharmacol* 39:203; 1990.
 56. Oppenheimer L, Capizzi TP, Weppleman RM, Mehta H: Determining the lowest limit of reliable assay measurement; *Anal Chem* 55:638; 1983.
 57. Paul BD, Past MR, McKinley RM, Foreman JD, McWhorter LK, Snyder JJ: Amphetamine as an artifact of methamphetamine during periodate degradation of interfering ephedrine, pseudoephedrine, and phenylpropanolamine: An improved procedure for accurate quantitation of amphetamines in urine; *J Anal Toxicol* 18:331; 1994.
 58. Pickup JF, McPherson K: Theoretical considerations in stable isotope dilution mass spectrometry for organic analysis; *Anal Chem* 48:1885; 1976.
 59. Podkowiak B-L, Repka ML, Smith ML: Interference of nitroindine in GC/MS confirmation of delta-9-THC-9-carboxylic acid in urine; *Clin Chem* 37:1305; 1991.
 60. Poole CF, Shuette S: *Contemporary Practice of Chromatography*; Elsevier: New York, NY; 1984.
 61. Provost LP, Elder RS: Interpretation of percent recovery data; *Am Lab* 15(12):57; 1983.
 62. Shewhart WA: *Statistical Method from the Viewpoint of Quality Control*; Dover Publications: New York, NY; 1986.
 63. Smith RV, Stewart JT: *Textbook of Biopharmaceutical Analysis*; Lea & Febiger: Philadelphia, PA; 1981.
 64. Sphon JA: Use of mass spectrometry for confirmation of animal drug residues; *J Assoc Off Anal Chem* 61:1247; 1978.
 65. Stevenson RY: An open letter to the food and drug administration; *Am Lab News Ed* 28(10):4; 1996.
 66. Tai SS-C, Christensen RG, Paule RC, Sander LC, Welch MJ: The certification of morphine and codeine in a human urine standard reference material; *J Anal Toxicol* 18:7; 1994.
 67. Thurman EM, Pedersen MJ, Stout RL, Martin T: Distinguishing sympathomimetic amines from amphetamine and methamphetamine in urine by gas chromatography/mass spectrometry; *J Anal Toxicol* 16:19; 1992.
 68. U.S. Department of Health and Human Services (Center for Drug Evaluation and Research, Food and Drug Administration): The draft guideline on validation of analytical procedures; *Fed Reg* 61:9315; 1996.
 69. U.S. Department of Health and Human Services (Center for Drug Evaluation and Research, Food and Drug Administration): *Reviewer Guidance — Validation of Chromatographic Methods*; 1994.
 70. U.S. Department of Health and Human Services (National Institute on Drug Abuse): Mandatory guidelines for federal workplace drug-testing programs; *Fed Reg* 53:11970; 1988.
 71. U.S. Department of Health and Human Services (National Laboratory Certification Program): Program Document #25; May 5, 1993.
 72. U.S. Department of Health and Human Services (Substance Abuse and Mental Health Services Administration): *Guidance Document for Laboratories and Inspectors*, Revised; August 29, 1994.
 73. U.S. Department of Health and Human Services (Center for Drug Evaluation and Research, Food and Drug Administration): *Guideline for Submitting Samples and Analytical Data for Methods Validation*; 1987.
 74. U.S. Department of Health and Human Services (Center for Drug Evaluation and Research, Food and Drug Administration): Draft guideline on validation of analytical procedures; *Fed Reg* 60:11260; 1995.
 75. Westgard JO: Better quality control through microcomputers; *Diagn Med* 61:741; 1982.
 76. Westgard JO, Barry PL: *Cost-Effective Quality Control: Managing The Quality and Productivity of Analytical Processes*; AACC Press: Washington, DC; 1986.
 77. Westgard JO, Barry PL, Hunt MR, Groth T: A multi-rule Shewhart chart for quality control in clinical chemistry; *Clin Chem* 27:493; 1981.
 78. Williams DR: An overview of test method validation; *BioPharm Manuf* 0:34; 1987.
 79. Wu AHB, Ostheimer D, Cremese M, Forte E, Hill D: Characterization of drug interferences caused by coelution of substances in GC/MS confirmation of targeted drugs in full-scan and selected-ion monitoring modes; *Clin Chem* 40:216; 1994.
 80. Wu Chen NB, Cody JT, Garriott JC, Foltz RL, Peat MA, Schaffer MI: Report of the 1988 Ad Hoc Committee on Forensic GC/MS: Recommended guidelines for forensic GC/MS procedures in toxicology laboratories associated with offices of medical examiners and/or coroners; *J Forensic Sci* 35:236; 1990.
 81. Yinon J: *Forensic Mass Spectrometry*; CRC Press: Boca Raton, FL; 1987.

ADDITIONAL READINGS

1. Aarons L: Effect of experimental design on assay calibration; *Analyst* 106:1249; 1981.
2. Bonate PL: Concepts in calibration theory. Part IV: Prediction and confidence intervals; *LC-GC* 10:531; 1992.
3. Burlingame AL, Boyd RK, Gaskell SJ: Mass spectrometry; *Anal Chem* 68:599R; 1996.
4. Chamberlain J: *Analysis of Drugs in Biological Fluids*, 2nd ed; CRC Press: Boca Raton, FL; 1995.

5. Freeman RR (Ed): *High Resolution Gas Chromatography*, 2nd ed; Hewlett-Packard: Wilmington, DE; 1981.
6. National Committee for Clinical Laboratory Standards: *Internal Quality Control Testing — Principles and Definitions, Approved Guidelines (C24-A)*; Villanova, PA; 1991.
7. Price P: Standard definitions of terms relating to mass spectrometry — A report from the Committee on Measurements and Standards of the American Society for Mass Spectrometry; *J Am Soc Mass Spectrom* 2:336; 1991.
8. Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Viswanathan CT, Cook CE: Analytical methods validation — Bioavailability, bioequivalence, and pharmacokinetic studies; *Eur J Drug Metab Pharmacokin* 16:249; 1991.
9. Taylor JK: *Quality Assurance of Chemical Measurements*; Lewis: Chelsea, MI; 1987.



Bruce A. Goldberger received a B.A. degree in zoology from Drew University (Madison, NJ) and M.S. and Ph.D. degrees in forensic toxicology from the School of Medicine at the University of Maryland in Baltimore (Baltimore, MD). Dr. Goldberger is currently an assistant professor and is director of toxicology in the Diagnostic Referral Laboratories of the Department of Pathology, Immunology, and Laboratory Medicine in the College of Medicine at the University of Florida (Gainesville, FL).

Dr. Goldberger is certified as a Toxicological Chemist by the National Registry in Clinical Chemistry and a Forensic Toxicology Specialist by the American Board of Forensic Toxicology. Dr. Goldberger was an assistant toxicologist and laboratory manager for the State of Maryland's Office of the Chief Medical Examiner and a toxicologist with the National Center for Forensic Science (Baltimore, MD). Dr. Goldberger's studies have included the analysis of alcohol in breath and the measurement of therapeutic and abused drugs in biological tissues. He has published numerous articles related to forensic toxicology and is co-editor (with Ray H. Liu of the University of Alabama at Birmingham) of the *Handbook of Workplace Drug Testing* (American Association for Clinical Chemistry Press: Washington, DC; 1995).

In recognition of his achievements in research in forensic toxicology, Dr. Goldberger was awarded the first annual Irving Sunshine Award from the Toxicology Section of the American Academy of Forensic Sciences (AAFS) in 1988. In addition, he was the 1994 recipient of the American Association for Clinical Chemistry's Outstanding Scientific Achievements by a Young Investigator Award. Dr. Goldberger is an active member of AAFS, American Association for Clinical Chemistry (AACC), National Safety Council's Committee on Alcohol and Other Drugs, and the Society of Forensic Toxicologists (SOFT).

Marilyn A. Huestis received a bachelor's degree in biochemistry from Mount Holyoke (South Hadley, MA), a master's degree in clinical chemistry from the University of New Mexico (Albuquerque, NM), and a doctoral degree in toxicology from the University of Maryland in Baltimore (Baltimore, MD), where she received a full research scholarship. Currently, Dr. Huestis is a senior research scientist at the Addiction Research Center (ARC), National Institute on Drug Abuse (NIDA), National Institute of Health (NIH), where her research interests include: (a) the pharmacokinetics and pharmacodynamics of drugs of abuse in humans, (b) evaluation of the effects of drugs on behavior including correlations of blood levels of drugs and concurrent changes in performance, (c) evaluation and validation of analytical techniques for detection of drugs of abuse in different biological fluids, and (d) medication development projects including the use of buprenorphine as a pharmacotherapeutic agent in opioid dependence. Dr. Huestis is interested in evaluating diverse routes of drug administration and determining the effect of different routes on the abuse liability of drugs, effects on performance, and on the onset, peak, and duration of action of the resultant effects.

Dr. Huestis has been working in the fields of forensic and analytical toxicology and clinical chemistry for more than 20 years. She was chief toxicologist of Nichols Institute's San Diego laboratory from 1983 to 1988, where she directed the emergency toxicology, therapeutic drug monitoring, analytical toxicology, and urine drug-testing operations. Dr. Huestis has published several important papers in the areas of marijuana pharmacokinetics and pharmacodynamics, in the evaluation and validation of immunoassays, and on the use of alternate testing matrices for drug analysis including hair, saliva, and sweat. She was nominated for the research staff fellow award of the ARC for her studies on the pharmacokinetics and pharmacodynamics of marijuana use. She has also been awarded the AAFS Irving Sunshine Award for outstanding research in forensic toxicology in 1992, and was elected to the Phi Kappa Phi Honor Society for outstanding academic achievement.

Goldberger, Huestis, and Wilkins • Quality Control Procedures for GC/MS Forensic Urine Drug Testing

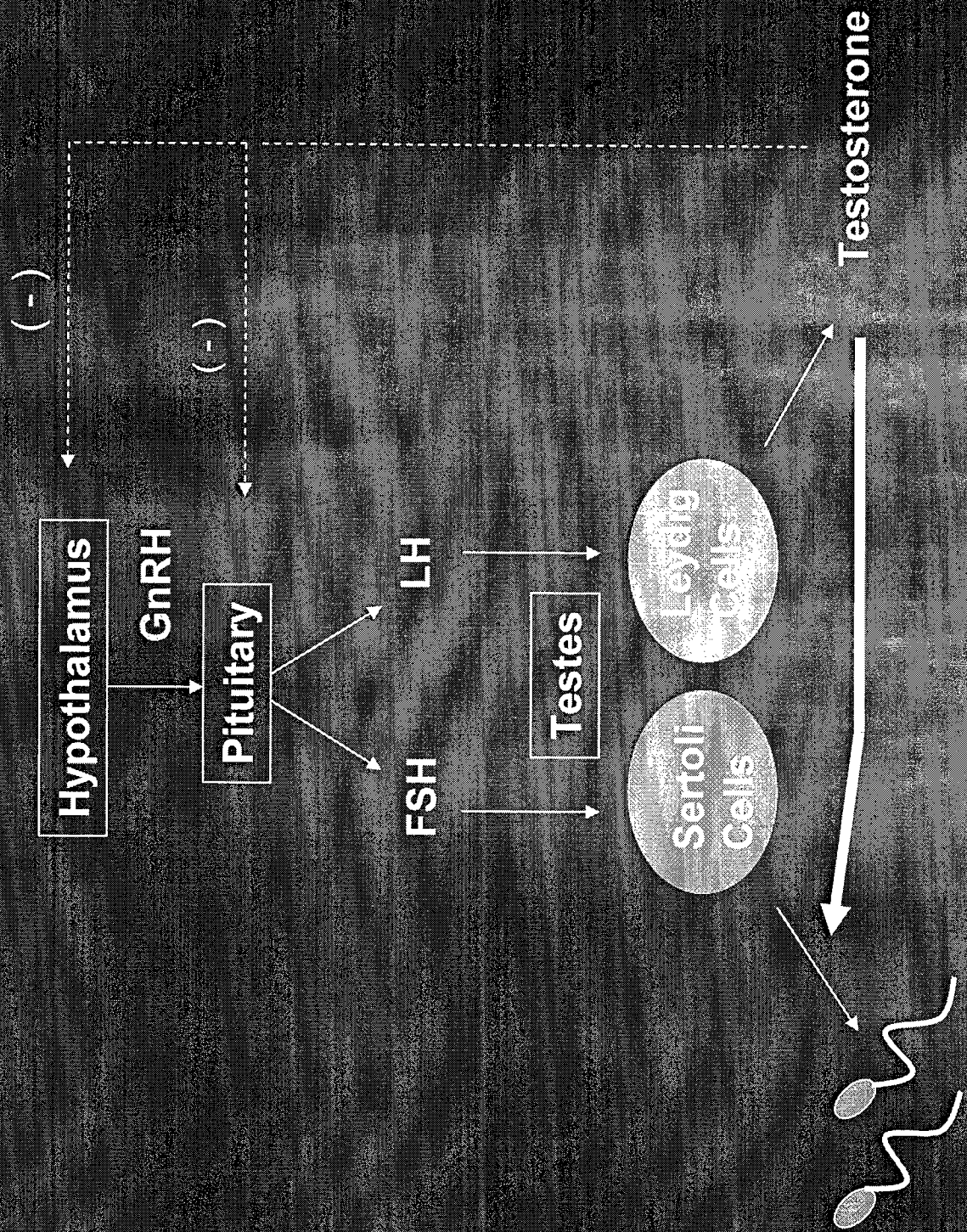
Dr. Huestis is on the Board of Directors of SOFT and currently serves as secretary of this organization. She also is program chair for the Toxicology Section of AAFS, is an active member and former secretary of the California Association of Toxicologists (CAT), serves on the Therapeutic Drug Monitoring and Clinical Toxicology Committee of AACC, and is a member of the International Association of Forensic Toxicology (TIAFT). Dr. Huestis was co-chair of the joint TIAFT/SOFT Joint Congress held in Tampa, FL in 1994.

Diana G. Wilkins received B.S. (1985), M.S. (1988), and Ph.D. (1991) degrees from the University of Utah (Salt Lake City, UT). She is currently assistant director of the Center for Human Toxicology at the University of Utah Health Sciences Center. She concurrently holds a faculty position as research assistant professor in the Department of Pharmacology and Toxicology, College of Pharmacy at the University of Utah.

Previously, Dr. Wilkins served as the quality control/quality assurance manager and senior toxicologist at the Center for Human Toxicology. Her postdoctoral education included training in toxicology and pharmacology, with particular emphasis on drugs of abuse. She currently participates in teaching undergraduate and graduate courses in clinical and analytical toxicology, pharmacology, and statistics for pharmacologic research at the University of Utah. Her research interests include investigation of the disposition of drugs of abuse in human hair and other biological matrices, as well as applications of mass spectrometry in biomedical research. She has obtained a research grant from NIDA to study the application of hair analysis to the monitoring of treatment compliance.

Dr. Wilkins is a laboratory inspector for the National Laboratory Certification Program under the auspices of the Department of Health and Human Services (HHS) and a laboratory assessor for the Standards Council of Canada. Dr. Wilkins was a member of the Drug Testing Advisory Board of the HHS Division of Workplace Performance for the period of 1994–1997. She has also served as an ad hoc member of several NIDA study sections. She belongs to several scientific associations, including AAFS, SOFT, TIAFT, CAT, and the Association for Women in Science.

Hormonal Control of Testicular Function



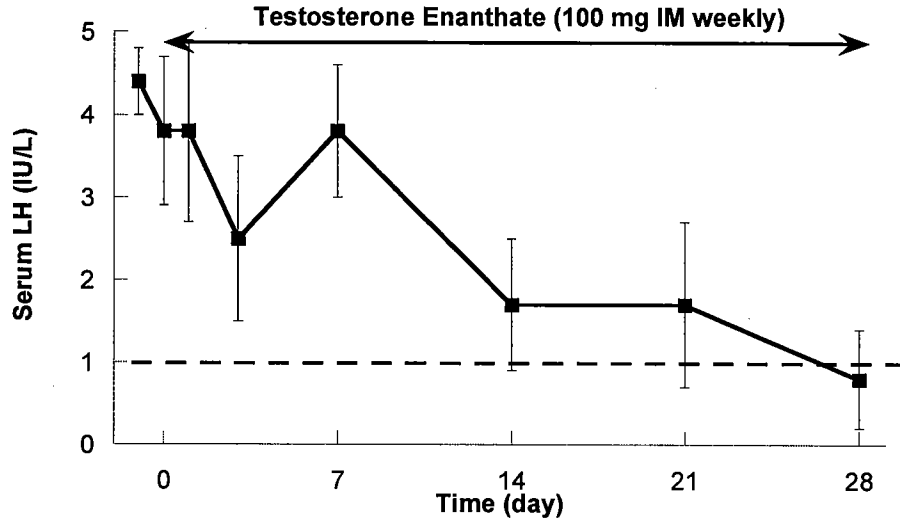


Figure. Serum luteinizing hormone (mean \pm SEM) prior to and during the administration of testosterone enanthate (100 mg IM q week) to seven normal men. The dotted line represents the lower limit of the normal range. Note that 4 weeks of testosterone administration are required to suppress serum luteinizing hormone release from the pituitary to abnormally low levels (Amory et al, manuscript in preparation).

Effect of multiple oral doses of androgenic anabolic steroids on endurance performance and serum indices of physical stress in healthy male subjects

Norbert Baume · Yorck Olaf Schumacher ·
Pierre-Edouard Sottas · Carlo Bagutti ·
Michel Cauderay · Patrice Mangin · Martial Saugy

Accepted: 4 July 2006 / Published online: 3 August 2006
© Springer-Verlag 2006

Abstract Anabolic androgenic steroids (AAS) are doping agents that are mostly used for improvement of strength and muscle hypertrophy. In some sports, athletes reported that the intake of AAS is associated with a better recovery, a higher training load capacity and therefore an increase in physical and mental performances. The purpose of this study was to evaluate, the effect of multiple doses of AAS on different physiological parameters that could indirectly relate the physical state of athletes during a hard endurance training program. In a double blind settings, three groups ($n = 9, 8$ and 8) were orally administered placebo, testosterone undecanoate or 19-norandrostenedione, 12 times during 1 month. Serum biomarkers (creatine kinase, ASAT and urea), serum hormone profiles (testosterone, cortisol and LH) and urinary catecholamines (noradrenalin, adrenalin and dopamine) were evaluated during the treatment. Running performance was assessed before and after the intervention phase by

means of a standardized treadmill test. None of the measured biochemical variables showed significant impact of AAS on physical stress level. Data from exercise testing on submaximal and maximal level did not reveal any performance differences between the three groups or their response to the treatment. In the present study, no effect of multiple oral doses of AAS on endurance performance or bioserum recovery markers was found.

Keywords Anabolic androgenic steroids · Doping · Recovery · Endurance training · Running

Introduction

The main aim of each professional or amateur athlete is to reach his maximal performance. To succeed, athletes need to balance between training loads (intensity and volume) and recovery periods which allow them to optimise their physiological and psychological capacities. Thus, training programs and specially recovery phases have to be well defined in order to avoid a prolonged imbalance that could lead to overreaching and/or overtraining (Halsen and Jeukendrup 2004; Hug et al. 2003; Kuipers and Keizer 1988; Lehmann et al. 1993; Petibois et al. 2002). In increasing the recovery capacities of the organism, the training volumes and intensities can be amplified thus reducing the risk to reach an overtraining state.

Recently, in endurance sports like cycling and athletics, some athletes reported anonymously the use of anabolic steroids, like testosterone and nandrolone, as an efficient exogenous aid to recover more rapidly following intense physical efforts (Hartgens and Kuipers 2004).

N. Baume (✉) · P. E. Sottas · P. Mangin · M. Saugy
Laboratoire Suisse d'Analyse du Dopage,
Institut Universitaire de Médecine Légale,
Ch. des Croisettes 22, 1066 Epalinges, Switzerland
e-mail: norbaume@gmail.com

Y. O. Schumacher
Abtlg. Sportmedizin, Medizinische Universitätsklinik
Freiburg, Hugstetter Strasse 55, 79106 Freiburg, Germany

C. Bagutti
Centre Médical Vidy-Med, Rte de Chavannes 11,
1000 Lausanne 23, Switzerland

M. Cauderay
Hôpital Riviera, Site du Samaritain, Bd. Paderewski 3,
1800 Vevey, Switzerland

The discovery of testosterone has given rise to the synthesis of multiple anabolic steroids (Kenyon et al. 1938). Rapidly, these products as well as testosterone have been used for doping purposes even if their performance-enhancing properties were largely inconclusive for many years (Haupt and Rovere 1984). Nevertheless, since 1980 some authors reported evidences that anabolic androgenic steroids (AAS) are effective in increasing muscle mass and improving strength (Bhasin et al. 1996; Friedl et al. 1991; Hartgens and Kuipers 2004) but the research done on the recovery rate in humans is too limited to draw definite conclusions yet. As recuperation is a physical and mental concept that is quite difficult to measure directly, the few research done in this context studied indirect parameters, like heart rate and serum lactate levels, creatinine, creatine kinase (CK) and aspartate aminotransferase (ASAT), associated with recovery time. Based on these criteria, some authors reported beneficial effects of AAS (Boone et al. 1990; Keul et al. 1976; Rozenek et al. 1990; Tamaki et al. 2001) whereas others found no consequence of AAS on recuperation (Jakob et al. 1988; Urhausen et al. 1989). Through experiments on birds, Alway and Starkweather (1993) suggested that the increased rate of protein synthesis due to AAS administration could be at the origin of a faster recovery from training load. An extrapolation was done from birds to humans without any scientific proofs.

As AAS are still at the origin of the major adverse analytical findings in the world antidoping laboratories (WADA 2005), the aim of the present study was to investigate more precisely the effect of multiple oral doses of testosterone undecanoate, 19-norandrostenedione or placebo on physical stress in male volunteers during a period of endurance training. Regarding ergogenics effects, the two investigated AAS can not be compared with others main AAS like metandienone or stanozolol which effects on muscle development and lean body mass have been clearly proven (Broeder 2003; Hartgens and Kuipers 2004).

Furthermore, we hypothesised that exogenous AAS intake could affect several blood and urinary parameters. Then some biological markers were analysed through the whole protocol in order to detect a potential typical profile of AAS doped athletes versus placebo. Hypothalamus-pituitary axis hormones (testosterone, cortisol, LH, hCG and SHBG) were measured to assess the effect of the administered AAS on blood hormones profile. Cardiac and muscular markers (Troponin I, Myoglobin, ALAT and Creatine Kinase) as well as inflammatory cytokine (IL-6) and catecholamines have been assessed to evaluate the

physiological effects of strenuous exercise in treated and placebo volunteers.

At the same time, running performances were measured through treadmill tests at the beginning and at the end of the protocol in order to put forward a positive global effect of testosterone and/or 19-norandrostenedione on physical adaptations to training.

Materials and methods

Selection of volunteers

The study was conducted at the Swiss Antidoping Laboratory, in Lausanne (Switzerland), after the approval of the protocol by the ethics committee from the Faculty of Medicine (University of Lausanne). Participants were healthy male volunteers from Caucasian origin, aged between 20 and 30, mainly from the Institute of Sports Sciences and Physical Education (University of Lausanne, Switzerland). All subjects practiced sports on a regular base with weekly training times ranging within 4 and 12 h. Thirty volunteers provided informed consent for the participation in the study. These students were not physically dependant on drugs and were instructed not to take any nutritional supplements or steroids 1 year before and during the study, which could affect their metabolism and steroid profile. Volunteers who were prone to hormonal dysfunction, cardiovascular disease, or have used controlled substances or any medication known to affect steroid metabolism were excluded from the study. Participants were evaluated medically (anamnesis and ECG at rest) and psychologically at the beginning of the study, before the treatment period, and at the end of the study in order to monitor their health. The anthropometrical characteristics of the participants as well as their physical level are summarized in Table 1. The fat percentage was determined with a four site (triceps, biceps, subscapular and suprailiac) skinfold measurement (Pongchaiyakul et al. 2005).

Exercise testing

The trial was divided in three phases. During the first part, all subjects performed a medical inclusive test and a running exercise test 1 month prior to the beginning of the treatment period. The exercise test was done on a ELM2 treadmill (Woodway, Weil am Rhein, Germany) where velocity was increased incrementally until subjective exhaustion of the tested subject. The initial workload was 6 km/h and running velocity was increased by 2 km/h at 3 min intervals. Blood lactate

Table 1 Mean anthropometrics values and physical performance (\pm SD) of the three groups at the beginning and at the end of the study

| | Placebo | | 19-Norandrostenedione | | Testosterone | |
|-----------------------------|-----------------|-----------------|-----------------------|-----------------|-----------------|-----------------|
| | Before | After | Before | After | Before | After |
| Age (years) | 24.2 \pm 2.3 | | 22.9 \pm 2.0 | | 24.1 \pm 1.6 | |
| Weight (kg) | 75.7 \pm 8.4 | 74.2 \pm 8.0 | 77.3 \pm 7.6 | 77.5 \pm 6.9 | 76.6 \pm 9.5 | 76.2 \pm 9.1 |
| Height (cm) | 182.4 \pm 4.5 | 182.4 \pm 4.5 | 183.4 \pm 5.9 | 183.4 \pm 5.9 | 182.7 \pm 6.6 | 182.7 \pm 6.6 |
| Fat mass (%) | 12.6 \pm 3.6 | 12.4 \pm 3.6 | 12.6 \pm 1.7 | 12.7 \pm 1.3 | 12.5 \pm 3.4 | 12.5 \pm 2.7 |
| Free fat mass (kg) | 66.0 \pm 6.1 | 64.9 \pm 5.6 | 67.5 \pm 6.8 | 67.6 \pm 6.1 | 66.9 \pm 6.8 | 66.6 \pm 6.9 |
| Body mass index | 20.7 \pm 2.0 | 20.3 \pm 1.8 | 21.0 \pm 1.5 | 21.1 \pm 1.3 | 21.0 \pm 2.2 | 20.8 \pm 2.0 |
| Running speed at IAT (km/h) | 13.3 \pm 1.9 | 14.4 \pm 1.8 | 12.7 \pm 1.5 | 14.0 \pm 0.7 | 13.6 \pm 0.7 | 14.7 \pm 1.2 |

concentrations at the different running velocities was measured with a Lactate Pro (Arkray, Kyoto, Japan) in capillary blood obtained during a 20 s-break after each stage from the hyperaemised earlobe. Heart rate was registered continuously using surface ECG during the last 30 s of each step of the protocol.

Training program

A special software (Ergonizer, Freiburg, Germany) was used for investigator-independent calculation of lactate threshold based on an equalizing SPLINE interpolation procedure (Roecker et al. 1998). As demarcations of intensity, heart rate at the lactate threshold (LT) and the so-called individual anaerobic threshold (IAT) were determined. The LT was taken from the original definition introduced by Wassermann et al. (1999) at the workload that corresponds to the start of the blood lactate concentration increase. IAT was set as the point at a net lactate increase of 1.5 mmol/l above LT (Dickhuth et al. 1991). Four individual training zones for each volunteer were defined based on the heart rate at the IAT as proposed by Roecker et al. (2002). Zone 1 (83–100% of LT) was used as exercise intensity for recovery or warm-up training. Zone 2 and 3 (100% of LT–95% of IAT for zone 2 and 95–101% of IAT for zone 3) determined the training intensity to improve aerobic performance. Training in zone 4 (99–107% of IAT) was used to increase the lactate tolerance (Roecker et al. 1997).

In order to level the performance of all participants, volunteers had to run four times a week at a low intensity included in the first or second training zone, 2 weeks before the first treatment. To manage their training during the protocol, a heart rate monitor (Fitwatch, Sigma Sport, Germany) was lent to the subjects.

Study medication

The second part of the study consisted in the oral intake of 12 placebo, 19-norandrostenedione or testos-

terone undecanoate pills on Monday, Wednesday and Friday between 6.30 and 8 am for 4 weeks (day 1–28). The subjects were randomly distributed in the three groups of ten individuals. The pills were composed of 300 mg of mannitol for placebo group (P group), 100 mg of 19-nor-4-androstenedione and 127 mg of mannitol for 19-norandrostenedione group (N group), 80 mg of testosterone undecanoate and 115 mg of mannitol for testosterone group (T group). Testosterone undecanoate (Dynapharm Distribution, Meyrin, Switzerland) and 19-nor-4-androstenedione (Sigma, St. Louis, MO, USA) were analytical tested by the Pharmacy of the University Hospital (CHUV, Lausanne, Switzerland) that also prepared the pills.

During the same time period, the volunteers were subjected to a strict individual training program (Fig. 1) aimed at improving endurance performance and causing fatigue and designed by expert staff. Meanwhile, all the subjects had to fill in the short version of POMS (Profile of Mood State) in order to assess the psychological profiles (data submitted for publication).

Spot urines were collected before the first pill intake, during the second week of the training period before and after the running sessions, on day 24 before treatment, 4, 8 and 24 h after and 13 days after the last pill intake (Fig. 1). Urines were kept at 4°C until their delivery to the laboratory and then each urine sample was divided into 20 ml flasks and stored at –20°C until extraction and analysis at the laboratory.

Blood analysis

Blood collections consisted in three tubes, one EDTA 2.7 ml and two serum 4.9 and 5.5 ml (Sarstedt AG & Co., Nümbrecht, Germany) withdrawn before the administration of the pill on day 1, 5, 8, 10, 12, 19, 26 and 29 in fasting conditions. The last tube was collected on day 38, i.e. 10 days after the end of the training program. The tubes were kept at 4°C (maximum 2 h) until the arrival at the laboratory. Hemograms were analysed on the EDTA tubes with a Sysmex XT 2000i

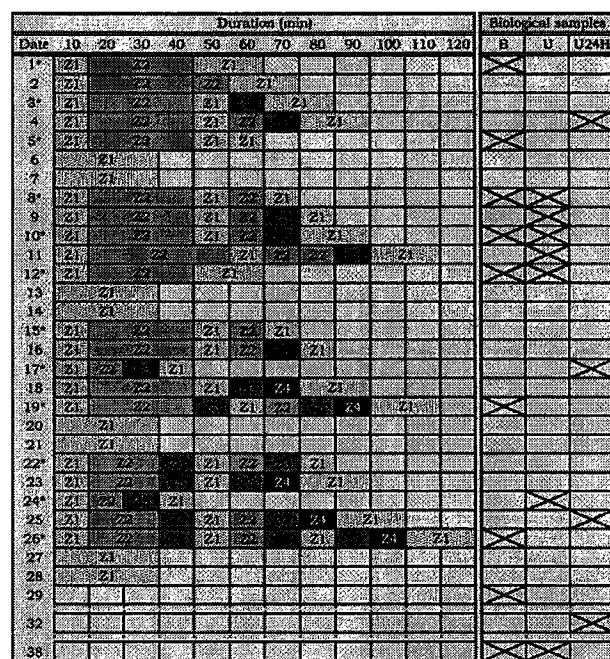


Fig. 1 Schematic representation of the main part of the protocol. The pill intakes are shown by the *asterisks*. The daily training program is detailed with the different training zones (*box with dots*, zone 1; *light grey shaded box*, zone 2; *dark grey shaded box*, zone 3 and *black box*, zone 4). The schedule of sampling is depicted on the *right side* (B, blood; U, urine; U24H: urines during 24 h)

(Sysmex Corporation, Kobe, Japan). These samples were then centrifuged 10 min at 1,700 g and plasma distributed in 3 ml polypropylene tubes and stored at -20°C until further analysis.

Serum tubes were directly centrifuged 10 min at 1,700 g and the plasma collected in 3 ml polypropylene tubes and stored at -20°C until analysis. Serum endocrinology parameters (cortisol, testosterone, LH, hCG, SHBG), muscle and cardiac biomarkers (Creatine Kinase, Troponin I, Myoglobine) and inflammatory markers (Interleukine-6) were measured with an Immulite 2000 (DPC Diagnostic Products Corporation, Los Angeles, CA, USA).

Aspartate, alanine aminotransferase (ASAT, ALAT) and urea were obtained from Dade Behring Diagnostics (Düdingen, Switzerland), runned on Dimension® RXL.

Catecholamines analysis

The subjects were asked to collect all their urines during 24 h on day 4, 17, 25 and 32 (U24H, see Fig. 1). Ten millilitres of HCl 5 N (Merck, Darmstadt, Germany) were added in the 3 l containers (Becton Dickinson BD, Franklin Lakes, USA) to stabilize the urines. Two

aliquots were taken in 10 ml urine monovette (Sarstedt AG & Co., Nümbrecht, Germany) and kept frozen at -20°C until analysis. Adrenalin, noradrenalin and dopamine were measured using high performance liquid chromatography (HPLC) with fluorescence detection (LC 240, Perkin Elmer, Boston, MA, USA).

Statistical analysis

All statistical analysis were performed on Matlab® version 6.1.0, with Statistics Toolbox version 3.0. Since distributions of the concentrations under study here are not necessarily normally distributed, a non-parametric one-way analysis of variance (Kruskal–Wallis ANOVA) was used for comparing means or medians between two groups of data. A *t*-test was also used to compare the sample average to a given constant. A significance level of $P = 0.05$ was considered.

Results

General considerations

Thirty male individuals were recruited to take part to this trial. Because of the vigorous training program imposed on the volunteers, five of them retired from the study due to physical problems (articulations and muscles soreness). The values measured on these individuals were not included in the analysis. The final repartition of subjects was 9, 8 and 8 individuals for P, T and N group, respectively. The anthropometrics values (Table 1) showed no statistical difference ($P > 0.05$) between the three groups before and at the end of the protocol.

The spot urines collected during the protocol were analysed for the detection of the endogenous urinary steroids. Isotope ratio mass spectrometry and gas chromatography coupled to mass spectrometry were used to detect variations of the testosterone metabolites compounds (Baume et al. 2006). Nandrolone metabolites (19-norandrosterone and 19-noretiocholanolone) stemming from the metabolism of 19-norandrostenedione (Bricout and Wright 2004) were also quantified in order to support previously published results (Baume et al. 2004) on the nandrolone metabolites pharmacokinetics (manuscript in preparation).

Physical performance

Three weeks before the first study medication, speed, heart rate, serum lactate concentration and exhaustion were determined during standardized treadmill test.

No statistical difference was found between the groups except for lactate I_{AT} and maximal speed between N and T groups ($P_{NT} = 0.02$ and 0.03). Statistical analysis were done on the same parameters established following a second treadmill test performed 10 days after the end of the training program. The results revealed no difference between the three groups ($P > 0.05$).

To put forward a potential beneficial effect of numerous oral doses of AAS on performance, the same physiological parameters were used. The intra-group differences between the post and the pre-treatment treadmill test values were calculated. As depicted in Fig. 2, a significant increase was observed for maximal speed ($\Delta_{\text{max speed}}$) in all groups (t -test for mean = 0; P_P, P_N and $P_T < 0.05$) and for the speed at the I_{AT} ($\Delta_{\text{speed } I_{AT}}$) for the N group. A noteworthy decrease was noted for the serum lactate concentration at the I_{AT} ($\Delta_{\text{lactate } I_{AT}}$) for P and N groups (P_P and $P_N < 0.05$).

Blood analysis

Several serum endocrinology parameters were measured. The time course of cortisol, total testosterone,

LH, T/LH and T/cortisol ratios along the protocol is depicted in Fig. 3. Comparison (Kruskal–Wallis analysis of variance) of values from the first and the last blood samples showed a significant decrease of cortisol for P and N groups ($P = 0.0007$ and $P = 0.0046$) and of total testosterone for the three groups ($P = 0.0041$, $P = 0.046$ and $P = 0.0063$, for P, N and T group, respectively). The T/LH ratio did not vary along the entire study (Kruskal–Wallis ANOVA; $P > 0.05$) but the T/Cortisol ratio increased significantly for the P group ($P = 0.01$). The first value measured before the beginning of treatment was compared to a group of values composed of the samples 2–8 (days 5, 8, 10, 12, 19, 26 and 29; i.e. during the treatment). No significant variation was noted for all the investigated parameters. The results of hCG analysis were mainly below the detection limit of the test used and are therefore not of use for any interpretation.

Cardiac biomarkers were also measured on all blood samples. Figure 4 shows the variations of creatine kinase. Additionally, the time course of ASAT and urea is depicted in same figure. The statistical analysis did not reveal any intragroup difference (Kruskal–Wallis ANOVA; $P > 0.05$). It has to be noticed that the CK data are expressed in ng/ml which is uncommon and

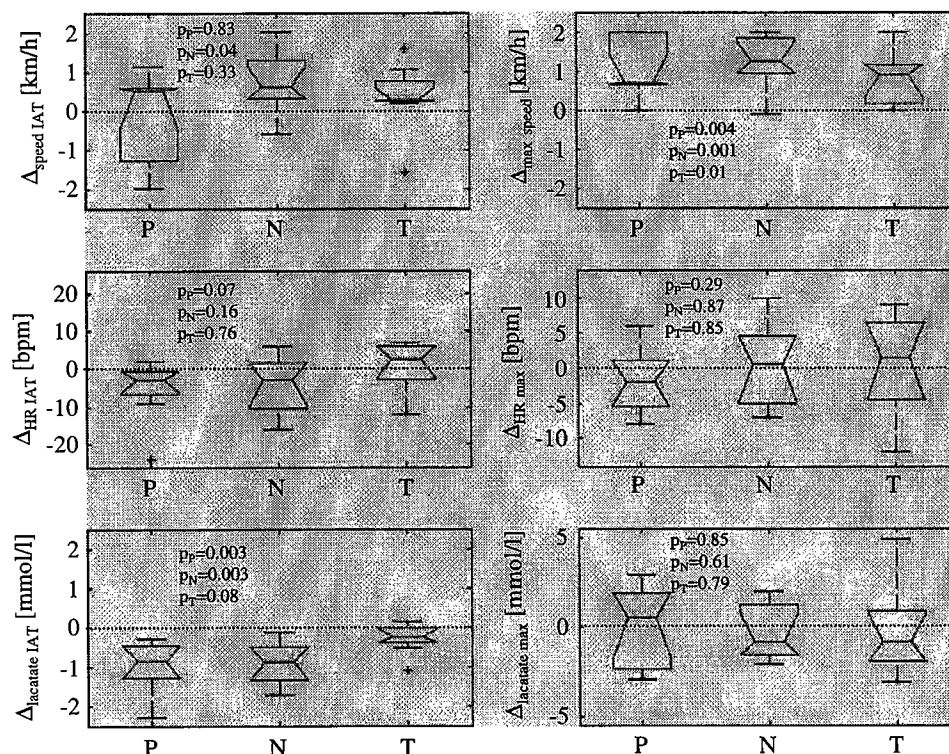


Fig. 2 Individual differences (Δ) between post and pre-treatment speed, heart rate (HR) and lactate values at the individual anaerobic threshold (I_{AT} , left part of the chart) and at exhaustion (max, right part of the chart) for the three groups placebo, 19-norandrostenedione and testosterone. P values indicate the probability of

a significant variation ($P < 0.05$) due to the administered product (i.e. P_T for the testosterone group). The boxes contain 25–75% of the values and the bars illustrate the 95% confidence interval. The median is represented by the line in the boxes. The crosses are the outliers

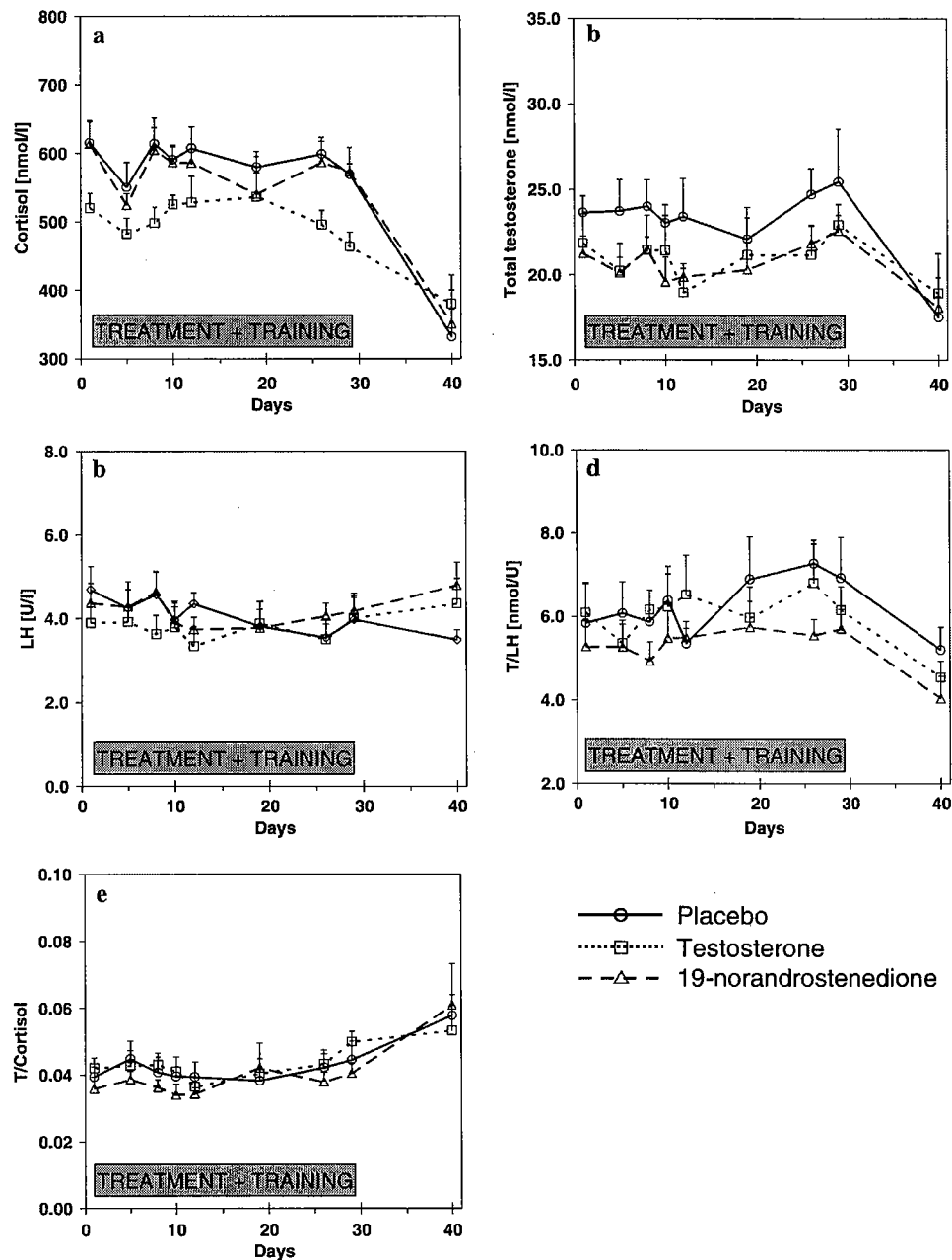


Fig. 3 Values of cortisol (a), total testosterone (b), LH (c), T/LH ratio (d) and T/Cortisol ratio (e) for the placebo (open circles, unbroken line), testosterone (open square, dotted line) and 19-no-

randrostenedione (open triangle, broken line) groups. Values are means \pm SEM. The training and treatment period (28 days, see Fig. 1) is indicated by the shadowed boxes

due to the measurement method. To put the data into perspective, a reference range study revealed a median value of 0.9 ng/ml, an upper 97.5th percentile of 2.6 ng/ml, and an upper 99 percentile of 4.8 ng/ml (Immulate 2000 CK-MB procedure, DPC, CA, USA).

As hCG, the detection of troponine I could not be used because of concentrations ranging below the detection limit of the test used. Myoglobin values remained invariant, the data are therefore not shown.

The blood parameters (full blood count including Hct, Hb, ...) measured on the EDTA tubes revealed no significant variation throughout the trial ($P > 0.05$) (data not shown).

Urinary catecholamines

Four times during the protocol, the volunteers had to collect their urines during 24 h (Fig. 1). The three

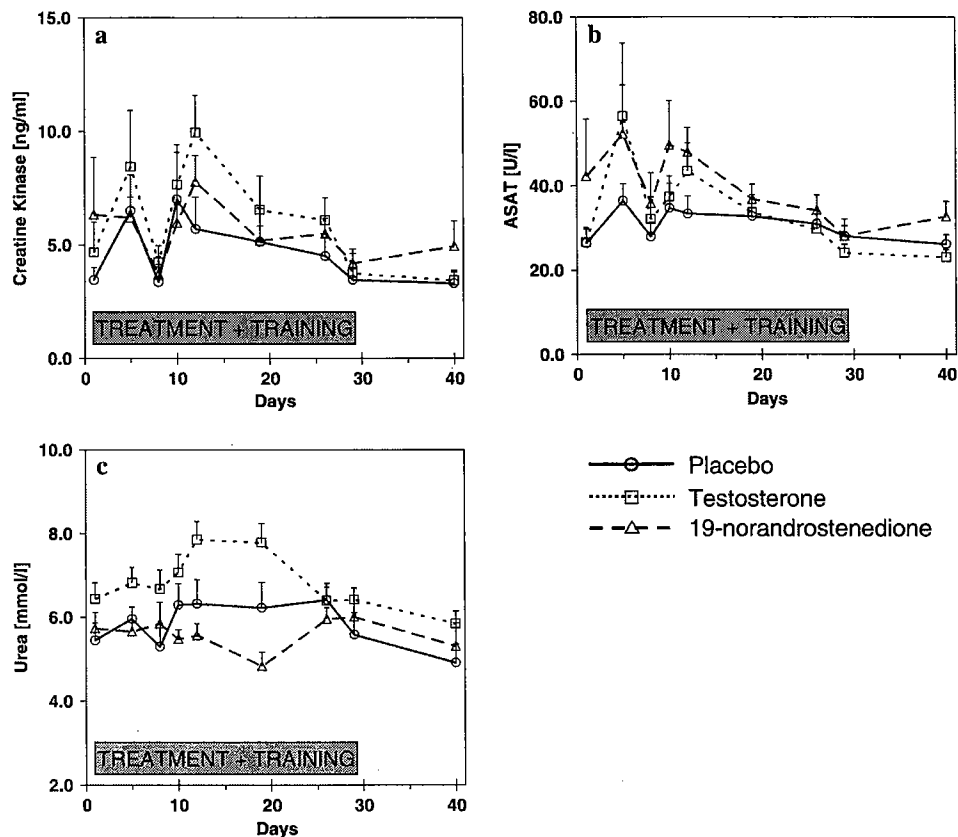


Fig. 4 Values of creatine kinase (a), ASAT (b) and urea (c) for the placebo (open circles, unbroken line), testosterone (open square, dotted line) and 19-norandrosteredione (open triangle,

broken line) groups. Values are means \pm SEM. As in Fig. 3, the training and treatment phase is indicated

major catecholamines, noradrenalin, adrenalin and dopamine were measured. The mean values of the four collection days are depicted in Fig. 5. The values are within the normal range (noradrenalin: 20–80 $\mu\text{g}/24\text{ h}$; adrenalin: 2–20 $\mu\text{g}/24\text{ h}$; dopamine: 190–450 $\mu\text{g}/24\text{ h}$). The only significant difference found during the test was for noradrenalin of the third urine (day 25) between P and T groups with a larger mean value for the T group ($P = 0.0045$).

Discussion

The purpose of this study was to evaluate a potential effect of multiple AAS oral administrations on running performances and on the physical stress level. Up to now, many studies investigated the influence of various AAS on muscular performance and recovery after strength and/or resistance exercise (Bhasin et al. 1996; Kadi 2000; Kuipers et al. 1993). To our knowledge, the endurance factor has never been studied in parallel with a chronic oral intake of testosterone and/or

nandrolone or its precursors. These two products were chosen because they seem to be the AAS most used in sports (WADA 2005). Moreover, oral treatments require an ester of testosterone (Wilson and Griffin 1980) because of the rapid hepatic metabolism of pure testosterone. Norandrosteredione is a recurrent contamination compound of nutritional supplements that are more and more used by athletes. For these reasons, the type of AAS and the doses used in our study could be considered as a good mimical representation of what might be used in professional and amateur sports. Nevertheless, the results of this study may not be directly transferred to all other AAS which could lead to more significant effects.

Exercise tests

To be able to assess a possible beneficial effect of AAS, the training protocol was specifically designed for endurance training based on training regimes used in the preparation of elite athletes and aimed at reaching a high level of physical and mental exhaustion at the

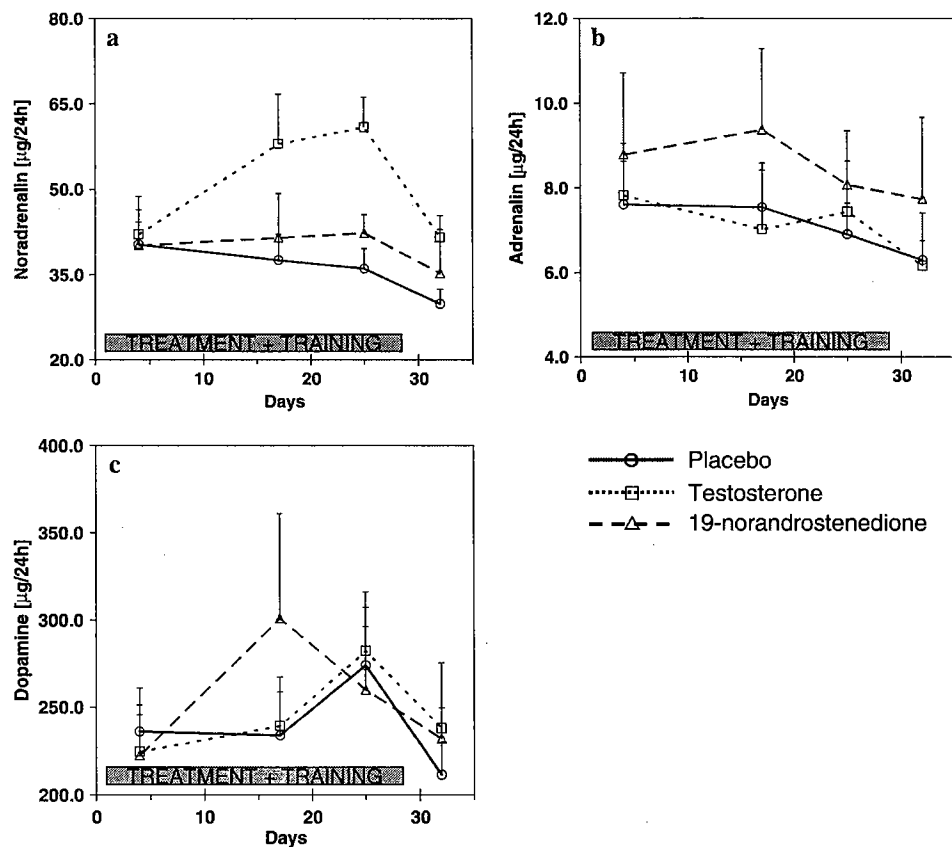


Fig. 5 Mean values (\pm SEM) of urinary excreted noradrenalin (a), adrenalin (b) and dopamine (c) for the placebo (open circles, unbroken line), testosterone (open square, dotted line) and

19-norandrostenedione (open triangle, broken line) groups. The training and treatment period is also depicted

end of the 4 weeks training in the volunteers (Fig. 1). This aim was well achieved according to the oral feedback obtained from the subjects.

For exercise testing, we used a protocol that has been used and validated for the evaluation of running performance in previous studies (Roeker et al. 1998). There are many physiological descriptors for the evaluation of performance. We choose the relation between running speed, serum lactate concentration and heart rate for the description of effort. From these parameters, a reliable prediction of race times in running competitions is possible (Roeker et al. 1998) and thus this test setting has a high practical impact for high performance sport. These three parameters were evaluated at two different time points during the treadmill tests. First, at the IAT and secondly at exhaustion (max) of the subjects. At the beginning of the protocol, only two values were found to be significantly different between N and T groups. T group subjects had a lactate concentration slightly under the one of the N group at the IAT and a maximal speed higher than N group individuals. These points toward the fact that the initial physical

level of the 25 individuals was not uniform and is a recurrent problem in the clinical studies made on human volunteers (Hartgens and Kuipers 2004) that is very difficult to avoid because of the high number of subjects that are required for such studies. Even if these differences exist, in the present study, there are quite negligible ($P = 0.02$ and 0.03). The P, T and N groups were therefore considered as comparable with each other. The variables measured during the treadmill tests performed after the training program (day 38) showed no variation between the three groups ($P > 0.05$) which can be interpreted as an identical intergroup response to training, regardless of the administered substances.

The intragroup performance variation following the training month was assessed in calculating the difference of the same values described above measured during the post and the pre-treatment treadmill tests (Fig. 2). Once again no clear conclusions can be drawn even if $\Delta_{\text{speed IAT}}$ of the N group subjects and $\Delta_{\text{lactate IAT}}$ for N and P groups significantly increased and decreased, respectively. Only one parameter ($\Delta_{\text{max speed}}$)

increased considerably for the three groups. It can be speculated that this increase of the maximal speed might be due to a uniform effect of the training program in the three groups.

On the other hand, there is an adaptation phenomenon of the subjects to the treadmill which implies that an individual will never perform two identical exercise tests. In addition, the intraindividual variation of the performance test might be greater than a potential effect of training or treatment. This is a recurrent problem in sports medicine (Hopkins 2004) and training planning. Therefore the differences observed between the performance related variables at the beginning and the end of the study are not important enough to prove that the investigated oral AAS are useful to increase endurance performance. It has to be noticed that the number of subjects in our study might be insufficient to unmask small effects of AAS. Indeed, it is possible that the performance is influenced by the oral intake of AAS in a range of 2–5%. Such a difference, e.g. in the speed at IAT might have a significant impact in a top level sporting competition but would require higher volunteer number to be revealed.

Hormone profiles

Many authors have investigated the effects of exercise on the endocrine system. While some studies demonstrated that physical efforts do not influence the concentrations of serum hormones like testosterone (Smilios et al. 2003), others clearly showed that there is a hormone variation (testosterone, LH and cortisol) due to exercise (Bonen et al. 1979; Galbo et al. 1977; Kuoppasalmi et al. 1980). These contradictory results could be explained by a reaction dependent on several factors like intensity, duration and mode of exercise and also by the training level of the subjects (Tremblay et al. 2004, 2005; Urhausen et al. 1995; Viru 1992). For this reason, the study protocol is a key criterion to consider before comparing the results reported in the literature. It is well documented that during and just after an endurance effort, testosterone (anabolic hormone) falls under the basal values whereas cortisol (catabolic hormone) concentration increases, which implies a decrease of the anabolic/catabolic (T/cortisol) ratio. It has been suggested that such adaptation allow amino acids to be redirected from protein synthesis to gluconeogenesis needed in stress situation (Duclos et al. 1996; Nindl et al. 2001). Up to now, only the immediate evolution (hours after efforts) of these biological markers has been investigated. With this study we focused on the middle and long-term changes of the hormonal profiles. The hormonal responses depicted in Fig. 3 show no significant

variation for total testosterone, cortisol and LH in the three groups. According to previously reported results (Fernandez-Garcia et al. 2002; Wheeler et al. 1991) a drop of the testosterone serum concentrations should have been observed but none of the three groups showed such picture. Moreover, the intake of testosterone undecanoate seems not to have any effect on the testosterone concentrations meaning that the oral doses were probably not sufficient enough to influence the hypothalamic-pituitary-testicular (HPT) axis. At the end of the training program, the volunteers could be considered as endurance trained athletes. Thus the final decline of testosterone, for the three groups, could be explained by a down regulation of the HPT axis that was already observed in this kind of athletes (Hackney et al. 1990, 1998). Free and bioactive testosterone concentrations were also calculated through the measurement of SHBG (data not shown). As this latter did not vary significantly, the free and bioactive testosterone profiles are the same as the one of total testosterone.

Cortisol has been proven to be one of the essential adaptation hormone during exercise (Viru and Viru 2004) but the variations are dependent of the intensity and duration of effort (Viru et al. 1992). As testosterone, cortisol serum concentration is quite stable during the treatment phase and drops significantly 10 days after the end of the training. The fact that cortisol levels did not change during the training was already observed by Keizer et al. (1989) and this might be explained by the rapid return of this hormone to the basal value. The final decrease of cortisol is probably an indicator of a complete recovery of the volunteers in whom the catabolic activity is not present any more.

Intuitively, longitudinal follow up of athletes during 1 month of endurance training should have resulted in a testosterone decline and then by a retro control feed back to a stimulation of the LH production (Matsumoto and Bremner 1984). As testosterone does not vary, LH remains stable during the entire study. These results have already been reported in the literature. MacConnie et al. (1986) stipulated that this non response of LH may be caused by the prolonged, repetitive elevations of gonadal steroids and other hormones known to suppress gonadotropin-releasing hormone secretion that are elicited by daily exercise. The T/LH serum and urinary ratios have been proven as an indicator of high injection dose of testosterone administration (Kicman et al. 1990). The multiple oral doses of testosterone undecanoate taken by the T groups subjects seem not be sufficient to have a significant impact on the HPT axis as testosterone and T/LH ratio are rather constant and not different from the two other groups, along the study.

All these results could indicate that the HPT axis has not been influenced by the duration (1 month) and intensity of the efforts. As the subjects trained outdoor, the conditions were not controlled as well as in laboratory settings. Nevertheless, the volunteers had to report their daily exercise (time of run and intensities) in order to allow a control of the protocol by the investigators. Except this control, the diet and others physical activities were not monitored. This is probably not an explanation of the relative hormonal stability which is most likely explained by the times of the blood withdrawals (between 6.30 and 8 am, 48 h after the previous medication).

Muscular activity biomarkers

To have an indirect measurement of the physical stress, the global time course of CK, ASAT and urea was assessed during the training month (Fig. 4). CK is an enzyme important for the muscular contraction cycle and present in striated and cardiac muscles. An augmentation of this biomarker could result from a release of the damaged muscles due to intense physical effort (Ebbeling and Clarkson 1989). The basal values of the three groups are slightly above the reference range values. This observation is common in people practicing sports on a regular base. Even if no statistical changes were observed for CK, the profile indicated that at the end of the two first training weeks (days 5 and 12) the subjects have highly solicited their muscles. On days 19 and 26, relatively low CK serum levels could express the organism adaptation to the training stress. ASAT is also contained in striated muscles and increase as well as CK after strenuous bout of exercise. The profile of ASAT is comparable with the one of the CK and confirms the observations made above. Urea is the final product of the amino acids degradation. The dosage of serum urea is mainly performed to evaluate the renal function which can be altered by intense physical exercise (Poortmans 1995). Once again, for testosterone group, the profile illustrates the exercise intensity but for N and P groups no clear pattern was detected. In the case of a better physical stress tolerance following the intake of oral AAS, it can be expected that one or more of these three biomarkers that were chosen for an indirect measurement of stress, would be more constant during an intense training program. Our results do not show any intergroup difference in the serum CK, ASAT or urea profiles. Subsequently, no beneficial effects on physical stress could be attributed to oral testosterone undecanoate or norandrostenedione.

Inflammatory and stress markers

IL-6 has been proven to be an inflammation responsive cytokine that drastically increase after strenuous exercise (Pedersen et al. 1998) and also after prolonged running (Ostrowski et al. 1999). Some authors postulated that IL-6 could be involved in hormonal response to training (Steensberg et al. 2003; Steinacker et al. 2004). The main part of IL-6 analysis performed on the serum samples were below the detection limit of the immunological test used and no statistical interpretation was possible. According to these results, we are not able to confirm an increase of IL-6 for both groups and this parameter seems therefore not useful for a long-term stress evaluation.

To have an indirect feed back of the physiological stress generated by the hard training, catecholamines were quantified in 24 h collected urines (Fig. 5). These are hormones secreted in stress situations. Changes in resting plasma and/or urinary catecholamines (adrenalin and noradrenalin) have been suggested as possible tools for monitoring the impact of training load and/or overload (Lehmann et al. 1997; Mackinnon et al. 1997). It has also been shown that excretion of catecholamines is correlated with training load (Atlaoui et al. 2006) and emotional stress (Lehmann et al. 1988). Between the three measured catecholamines, only noradrenalin seems to be influenced by effort as an increase is observed for the T group. Even if this is a significant change, no reasonable explanation was found.

Limitations

Some limitations must be taken into consideration. First, the fact that training and treatment are done at the same time complicated the interpretation of the results. Second, the statistical models used are of limited significance for a small number of volunteers. More subjects could have unmasked differences in a range of 2–5%. However, small differences would be of great importance for professional athletes that compete at the highest level. Our observations are not in accordance with the major studies made in the field of physical stress and hormone response following endurance exercise, probably because contrasting most other studies, we tried to investigate the development of hormones and other physiological markers in a time period of days rather than hours. The authors estimate that the training intensity and volume were sufficient to trigger typical hormonal responses. Third, the doses of AAS administered in our investigation could have been too low to observe any performance or recovery improvements. However,

our doses were supposed to mimic the ones taken by athletes and higher amounts would not be realistic in the antidoping field. Maybe that a prolonged medication period could lead to statistical differences regarding the investigated physiological parameters between AAS groups and placebo. The effect of AAS could also be psychological and, in that case might not be measurable through physiological parameters.

Conclusion

This study investigated the effect of multiple oral AAS intake on performance capacity and markers of physical stress of endurance trained athletes. Based on the results, it is not obvious that a regular exogenous supply of testosterone undecanoate or 19-norandrostenedione (a nandrolone precursor) reduces physical stress or improves endurance performance of the athlete.

According to these observations, further investigations with higher number of subjects and different treatment times are needed to support the hypothesis that AAS have a real impact on the organism to improve recovery during and after endurance efforts.

Acknowledgements We would like to acknowledge the Federal Office of Sports (BASPO) of Switzerland which provided the financial support for this study and also the 30 participants coming mainly from the Institute of Sport Sciences and Physical Education (ISSEP, University of Lausanne). The authors are also grateful towards Mr Rodolphe Gerber from Bühlmann Laboratories AG (Schönenbuch, Switzerland), Polyanalytic SA (Morges, Switzerland) and the department of sport medicine of the clinical University of Freiburg (Germany) for their technical support for the blood and urine sample analysis. The investigators are thankful towards Sigma Sport (Germany) for having lent the heart rate monitors (Fitwatch).

References

- Alway SE, Starkweather LA (1993) Effect of anabolic steroids on new fiber formation and fiber area during stretch-overload. *J Appl Physiol* 74:832–837
- Atlaoui D, Duclos M, Gouarne C, Lacoste L, Barale F, Chatard JC (2006) 24-hr urinary catecholamine excretion, training and performance in elite swimmers. *Int J Sports Med* 27(4):314–321
- Baume N, Avois L, Schweizer C, Cardis C, Dvorak J, Cauderay M, Mangin P, Saugy M (2004) [¹³C] Nandrolone excretion in trained athletes: interindividual variability in metabolism. *Clin Chem* 50:355–364
- Baume N, Saudan C, Desmarchelier A, Strahm E, Sottas PE, Bagutti C, Cauderay M, Schumacher YO, Mangin P, Saugy M (2006) Use of isotope ratio mass spectrometry to detect doping with oral testosterone undecanoate: Inter-individual variability of (13)C/(12)C ratio. *Steroids* (in press)
- Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Tricker R, Shirazi A, Casaburi R (1996) The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med* 335:1–7
- Bonen A, Ling WY, MacIntyre KP, Neil R, McGrail JC, Belcastro AN (1979) Effects of exercise on the serum concentrations of FSH, LH, progesterone, and estradiol. *Eur J Appl Physiol Occup Physiol* 42:15–23
- Boone JB Jr, Lambert CP, Flynn MG, Michaud TJ, Rodriguez-Zayas JA, Andres FF (1990) Resistance exercise effects on plasma cortisol, testosterone and creatine kinase activity in anabolic-androgenic steroid users. *Int J Sports Med* 11:293–297
- Bricout V, Wright F (2004) Update on nandrolone and norsteroids: how endogenous or xenobiotic are these substances? *Eur J Appl Physiol* 92:1–12
- Broeder CE (2003) Oral andro-related prohormone supplementation: do the potential risks outweigh the benefits? *Can J Appl Physiol* 28(1):102–116
- Dickhuth HH, Huonker M, Münzel T, Drexler A, Berg A, Keul J (1991) Individual anaerobic threshold for the evaluation of competitive athletes and patients with left ventricular dysfunction. In: Bachl TG, Löllgen H (eds) *Advances in ergometry*. Springer, Berlin Heidelberg New York, pp 173–179
- Duclos M, Corcuff JB, Rashedi M, Fougere V, Manier G (1996) Does functional alteration of the gonadotropic axis occur in endurance trained athletes during and after exercise? A preliminary study. *Eur J Appl Physiol Occup Physiol* 73:427–433
- Ebbeling CB, Clarkson PM (1989) Exercise-induced muscle damage and adaptation. *Sports Med* 7:207–234
- Fernandez-Garcia B, Lucia A, Hoyos J, Chicharro JL, Rodriguez-Alonso M, Bandres F, Terrados N (2002) The response of sexual and stress hormones of male pro-cyclists during continuous intense competition. *Int J Sports Med* 23:555–560
- Friedl KE, Dettori JR, Hannan CJ Jr, Patience TH, Plymate SR (1991) Comparison of the effects of high dose testosterone and 19-nortestosterone to a replacement dose of testosterone on strength and body composition in normal men. *J Steroid Biochem Mol Biol* 40:607–612
- Galbo H, Hummer L, Peterson IB, Christensen NJ, Bie N (1977) Thyroid and testicular hormone responses to graded and prolonged exercise in man. *Eur J Appl Physiol Occup Physiol* 36:101–106
- Hackney AC, Sinning WE, Bruot BC (1990) Hypothalamic-pituitary-testicular axis function in endurance-trained males. *Int J Sports Med* 11:298–303
- Hackney AC, Fahrner CL, Gullledge TP (1998) Basal reproductive hormonal profiles are altered in endurance trained men. *J Sports Med Phys Fitness* 38:138–141
- Halsen SL, Jeukendrup AE (2004) Does overtraining exist? An analysis of overreaching and overtraining research. *Sports Med* 34(14):967–981
- Hartgens F, Kuipers H (2004) Effects of androgenic-anabolic steroids in athletes. *Sports Med* 34:513–554
- Haupt HA, Rovere GD (1984) Anabolic steroids: a review of the literature. *Am J Sports Med* 12:469–484
- Hopkins W (2004) How to interpret changes in an athletic performance test. *Sportscience* 8:1–7. <http://www.sportsci.org/jour/04/wghtests.htm>. Accessed Sept 25, 2005
- Hug M, Mullis PE, Vogt M, Ventura N, Hoppeler H (2003) Training modalities: over-reaching and over-training in athletes, including a study of the role of hormones. *Best Pract Res Clin Endocrinol Metab* 17:191–209
- Jakob E, Hoffman R, Fuchs F, Stüwe-Schlobies M, Donike M, Keul J (1988) Testosteronapplikation und Leistungsfähigkeit bei Skilangläufern. *Dtsch Z Sport Med* 39:41–45
- Kadi F (2000) Adaptation of human skeletal muscle to training and anabolic steroids. *Acta Physiol Scand* 646(Suppl):1–52

- Keizer H, Janssen GM, Menheere P, Kranenburg G (1989) Changes in basal plasma testosterone, cortisol, and dehydroepiandrosterone sulfate in previously untrained males and females preparing for a marathon. *Int J Sports Med* 10(Suppl 3):S139–S145
- Kenyon AT, Sandiford I, Bryan AM, Knowlton K, Koch FC (1938) The effect of testosterone propionate on nitrogen, electrolytes, water and energy metabolism in eunuchoidism. *Endocrinology* 23:135–144
- Keul J, Deus B, Kindermann W (1976) Anabole hormone: Schädigung, Leistungsfähigkeit und Stoffwechsel. *Med Klin* 71:497–503
- Kicman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee MN, Southan GJ, Wheeler MJ (1990) Criteria to indicate testosterone administration. *Br J Sports Med* 24:253–264
- Kuipers H, Keizer HA (1988) Overtraining in elite athletes. Review and directions for the future. *Sports Med* 6:79–92
- Kuipers H, Peeze Binkhorst FM, Hartgens F, Wijnen JA, Keizer HA (1993) Muscle ultrastructure after strength training with placebo or anabolic steroid. *Can J Appl Physiol* 18:189–196
- Kuoppasalmi K, Naveri H, Harkonen M, Adlercreutz H (1980) Plasma cortisol, androstenedione, testosterone and luteinizing hormone in running exercise of different intensities. *Scand J Clin Lab Invest* 40(5):403–409
- Lehmann M, Jakob E, Roscher E, Tusch R, Keul J (1988) Ski-flying: related catecholamin excretion compared with cross-country skiing. *Int J Sports Med* 9:438–442
- Lehmann M, Foster C, Keul J (1993) Overtraining in endurance athletes: a brief review. *Med Sci Sports Exerc* 25:854–862
- Lehmann MJ, Lormes W, Opitz-Gress A, Steinacker JM, Netzer N, Foster C, Gastmann U (1997) Training and overtraining: an overview and experimental results in endurance sports. *J Sports Med Phys Fitness* 37:7–17
- MacConnie SE, Barkan A, Lampman RM, Schork MA, Beitins IZ (1986) Decreased hypothalamic gonadotropin-releasing hormone secretion in male marathon runners. *N Engl J Med* 315:411–417
- Mackinnon LT, Hooper SL, Jones S, Gordon RD, Bachmann AW (1997) Hormonal, immunological, and hematological responses to intensified training in elite swimmers. *Med Sci Sports Exerc* 29:1637–1645
- Matsumoto AM, Bremner WJ (1984) Modulation of pulsatile gonadotropin secretion by testosterone in man. *J Clin Endocrinol Metab* 58(4):609–614
- Nindl BC, Kraemer WJ, Deaver DR, Peters JL, Marx JO, Heckman JT, Loomis GA (2001) LH secretion and testosterone concentrations are blunted after resistance exercise in men. *J Appl Physiol* 91:1251–1258
- Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK (1999) Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol* 515:287–291
- Pedersen BK, Ostrowski K, Rohde T, Bruunsgaard H (1998) The cytokine response to strenuous exercise. *Can J Physiol Pharmacol* 76:505–511
- Petibois C, Cazorla G, Poortmans JR, Deleris G (2002) Biochemical aspects of overtraining in endurance sports: a review. *Sports Med* 32(13):867–878
- Pongchaiyakul C, Kosulwat V, Rojroongwasinkul N, Charoenkiatkul S, Thepsuthammarat K, Laopaiboon M, Nguyen TV, Rajatanavin R (2005) Prediction of percentage body fat in rural Thai population using simple anthropometric measurements. *Obes Res* 13(4):729–738
- Poortmans JR (1995) Renal response to exercise in healthy and diseased patients. *Nephrologie* 16:317–324
- Röcker K, Schotte O, Niess A, Heitkamp HC, Dickhuth HH (1997) Laufbandtestdaten und Wettkampfprognosen für den Langstreckenlauf. *D Z Sportmed* 48:315–323
- Roecker K, Schotte O, Niess AM, Horstmann T, Dickhuth HH (1998) Predicting competition performance in long-distance running by means of a treadmill test. *Med Sci Sports Exerc* 30:1552–1557
- Roecker K, Niess AM, Horstmann T, Striegel H, Mayer F, Dickhuth HH (2002) Heart rate prescriptions from performance and anthropometrical characteristics. *Med Sci Sports Exerc* 34:881–887
- Rozenek R, Rahe CH, Kohl HH, Marple DN, Wilson GD, Stone MH (1990) Physiological responses to resistance-exercise in athletes self-administering anabolic steroids. *J Sports Med Phys Fitness* 30:354–360
- Smilios I, Piliandis T, Karamouzis M, Tokmakidis SP (2003) Hormonal responses after various resistance exercise protocols. *Med Sci Sports Exerc* 35:644–654
- Steensberg A, Fischer CP, Keller C, Møller K, Pedersen BK (2003) IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am J Physiol Endocrinol Metab* 285:E433–E437
- Steinacker JM, Lormes W, Reissnacker S, Liu Y (2004) New aspects of the hormone and cytokine response to training. *Eur J Appl Physiol* 91:382–391
- Tamaki T, Uchiyama S, Uchiyama Y, Akatsuka A, Roy RR, Edgerton VR (2001) Anabolic steroids increase exercise tolerance. *Am J Physiol Endocrinol Metab* 280:E973–E981
- Tremblay MS, Copeland JL, Van Helder W (2004) Effect of training status and exercise mode on endogenous steroid hormones in men. *J Appl Physiol* 96:531–539
- Tremblay MS, Copeland JL, Van Helder W (2005) Influence of exercise duration on post-exercise steroid hormone responses in trained males. *Eur J Appl Physiol* 94:505–513
- Urhausen A, Stein R, Kindermann W (1989) Metabolismus und hormonelles Verhalten bei Ausdauertrainierten unter Testosteronapplikation. *Dtsch Z Sport Med* 40:312–320
- Urhausen A, Gabriel H, Kindermann W (1995) Blood hormones as markers of training stress and overtraining. *Sports Med* 20:251–276
- Viru A (1992) Plasma hormones and physical exercise. *Int J Sports Med* 13:201–209
- Viru A, Viru M (2004) Cortisol—essential adaptation hormone in exercise. *Int J Sports Med* 25:461–464
- Viru A, Karelson K, Smirnova T (1992) Stability and variability in hormonal responses to prolonged exercise. *Int J Sports Med* 13:230–235
- WADA, The World Anti-Doping Agency (2005) 2004 Adverse analytical findings reported by Accredited Laboratories, May 2005. <http://www.wada-ama.org>
- Wasserman K, Hansen JE, Sue DY, Stringer WW, Whipp BJ (1999) Principles of exercise testing and interpretation, 4th edn. Lippincott, Williams & Wilkins, Baltimore, pp 568
- Wheeler GD, Singh M, Pierce WD, Epling WF, Cumming DC (1991) Endurance training decreases serum testosterone levels in men without change in luteinizing hormone pulsatile release. *J Clin Endocrinol Metab* 72:422–425
- Wilson JD, Griffin JE (1980) The use and misuse of androgens. *Metabolism* 29:1278–1295

Exogenous testosterone, aggression, and mood in eugonadal and hypogonadal men

Daryl B. O'Connor^{a,*}, John Archer^b, W. Morton Hair^a, Frederick C.W. Wu^a

^a*Department of Endocrinology, Manchester Royal Infirmary, Manchester M13 9WL, UK*

^b*Department of Psychology, University of Central Lancashire, Preston, Lancashire PR1 2HE, UK*

Received 19 July 2001; received in revised form 8 October 2001; accepted 15 January 2002

Abstract

To investigate (1) the effects of exogenous testosterone (T) on self- and partner-reported aggression and mood and (2) the role of trait impulsivity in the T–aggression relationship. Thirty eugonadal men with partners were randomized into two treatment groups to receive: (1) 200 mg im T enanthate weekly for 8 weeks or (2) 200 mg im sodium chloride weekly for 8 weeks. Eight hypogonadal men received 200 mg im T enanthate biweekly for 8 weeks. All groups completed a battery of behavior measures at baseline (Week 0) and at Weeks 4 and 8. Cognitive and motor impulsivity were the only predictors of self-reported total aggression (over and above age and T levels) at Weeks 0, 4, and 8. No significant changes in aggression or mood levels were found in the eugonadal-treated group. Significant reductions in negative mood (tension, anger, and fatigue) followed by an increase in vigor were found in response to T treatment in the hypogonadal group. These results demonstrate that inability to control one's behavior when such control is required by a particular situation (impulsivity) was found to significantly predict levels of aggression over and above age and T level. These data do not support the hypothesis that supraphysiological levels of T (within this range) lead to an increase in self- and partner-reported aggression or mood disturbances. Instead, for the first time, this study has identified the high level of negative affect experienced by hypogonadal patients. These findings have implications for T replacement therapy and male contraception. © 2002 Published by Elsevier Science Inc.

Keywords: Testosterone; Aggression; Hostility; Impulsivity; Hypogonadal; Eugonadal

1. Introduction

The effect of testosterone (T) on behavior, and in particular aggression, has received considerable attention over the last decade [1–4]. This has been prompted by several developments. Firstly, the high-profile media coverage of incidents of 'steroid rage' seemingly associated with the abuse by strength athletes of androgenic–anabolic steroids (AAS) [5]. Secondly, the use of exogenous T clinically as part of the development of a reversible, hormonal contraceptive for men [6], its use for replacement therapy in HIV illness [7], and for treating the psychological and physiological effects of aging in men [8].

In general, these studies can be characterised as: (1) Comparisons between violent criminals and nonviolent controls on some measure of aggression [9–11]. These studies

generally demonstrate higher T levels among the more violent men than the less violent ones. (2) Correlational studies investigating the degree of association among endogenous T levels, aggression, and hostile feelings. These studies have shown conflicting results, with stronger associations being found when behavioral and observational measures have been employed compared to trait measures of aggression [9,12,13]. (3) Experimental studies where T levels have been manipulated into the supraphysiological range [1,14–17]. The existing experimental studies have yielded varied results, and they are difficult to interpret owing to differences in experimental manipulations and in the dependent variables.

Here, we examine reports providing measures of direct aggression for baseline or placebo phases, and for phases when levels of T were elevated to supraphysiological levels. We calculated effect sizes (*g*) from the means and standard deviations provided in the papers, for the differences between baseline and T conditions, and for the differences between placebo and T conditions [18]. Su et al. [16]

* Corresponding author. School of Psychology, University of Leeds, Leeds, LS2 9JT, UK. Tel.: +44-113-233-5727; fax: +44-113-233-5749.

E-mail address: daryl@psychology.leeds.ac.uk (D.B. O'Connor).

injected 20 men with placebo, followed by a low dose of methyltestosterone, and then a high-dose methyltestosterone. Their psychological measures were mainly concerned with mood, but included ratings of 'violent feelings' on a visual analogue scale. Comparing baseline with the high-dose phase yielded a value of $g=.28$; comparing baseline with the low-dose phase yielded a value of $g=.09$. However, the authors report trends for an increase in anger and violent feelings in the high-dose group. Yates et al. [17] reported a double-blind study of 31 men involving 100, 250, and 500 mg of T cypionate. For self-ratings on the assault scale of the Buss–Durkee Hostility Inventory (BDHI), values indicated no difference between baseline or placebo and the average of all three doses of T ($g=.08$ for baseline and $g=0$ for placebo). However, values indicating an increase on this scale were found when the ratings were provided by an informant rather than the person himself ($g=.47$ for comparisons with baseline and $g=.17$ for placebo). Pope et al. [15] reported values on a laboratory measure of aggression [the Point Subtraction Aggressive Paradigm (PSAP) by Cherek et al. [19]] for baseline, placebo, and treatment phases for 27 men receiving up to 600 mg T cypionate. The values were $g=.57$ for the baseline comparison and $g=.51$ for the treatment comparison, indicating a rise in aggression following T treatment. Although the Aggression Questionnaire (AQ), the modern version of the BDHI, was used in this study the values presented were aggregates for the four subscales: physical and verbal aggression, hostility, and anger. An initial report based on 8 of the 51 men completing this measure [20] indicated an increase in physical aggression following treatment ($g=.79$), but a much smaller one for verbal aggression ($g=.20$). In the complete sample, the values for the total AQ scores were around zero, indicating no change in this composite measure of direct aggression, hostility, and anger.

Over the three studies, when effect sizes are calculated, there is some evidence for increases in measures of direct aggression following T administration, but this is inconsistent, and nonsignificant in most cases.

Experimental studies have generally not found evidence for a T–anger relationship [1,14,21]. For example, Tricker et al. [1] administered a large supraphysiological dose of T enanthate (600 mg/week) to a sample of healthy men in a double-blind, placebo-controlled fashion and assessed both self-reported and partner-reported mood and anger. Their study failed to detect any effects on angry behavior or mood. More intriguingly, Bjorkqvist et al. [21] found evidence for a placebo effect, where men who received a placebo in a double-blind experiment reported higher scores on self-estimated anger, irritation, impulsivity, and frustration than those receiving T orally (Panteston). These increases were also supported by the observer estimates. However, these findings should be interpreted with caution given that the orally administered T did not increase circulating T levels into the supraphysiological range.

One concern in this area [21] is that positive results are more readily reported than null ones and that investigators are less likely to submit manuscripts that accept null hypotheses. These findings also suggest that the relationship between T (or steroid use) and aggressive behavior may not be a direct, causal one. With this in mind, it is important to note that few experimental studies have investigated the possible role of individual difference variables, such as impulsivity, in their experimental paradigms [3]. Research in this area is also limited by over-reliance on self-report data, with few studies incorporating observations from a partner or significant other (e.g. Ref. [1]). There is also a dearth of controlled investigations of the effects of T administration on aggressive, hostile behavior, and mood in young hypogonadal men who have low levels of T [22].

The aim of this study was to investigate the dose–response relationship among T levels, behavior, and mood in men. A eugonadal-treated group receiving weekly injections of supraphysiological levels of T and a hypogonadal group receiving physiological replacement levels of T were compared with a eugonadal-placebo control group, over an 8-week period. To improve on previous studies, a wide range of measures were used, including self- and partner-reported aggression, anger and hostility, self-esteem, assertiveness, irritability, and moods. Trait impulsivity was also assessed at baseline.

2. Method

2.1. Subjects

Thirty healthy male volunteers (mean age = 28.2 years; range 19–45 years) and 8 hypogonadal male patients (mean age = 30.8 years; range 23–40 years) participated in the study. The eugonadal men were recruited from local radio and newspaper advertisements. The hypogonadal patients were recruited from the Department of Endocrinology, Manchester Royal Infirmary and Hope Hospital, Manchester, UK. The hypogonadal group consisted of three men with Kallmann's syndrome, two with Klinefelter's syndrome, and three who had a bilateral orchidectomy receiving maintenance T replacement for a mean of 5 years (range 0–14 years).

2.2. Design

Volunteers who met the admission criteria after medical screening were randomised into two treatment groups ($n=15$) to receive (1) in the eugonadal-treated group: 200 mg im T enanthate (Cambridge Laboratories, UK) once weekly for 8 weeks or (2) in the eugonadal-placebo group: 200 mg im 0.9% sodium chloride solution weekly for 8 weeks. The eugonadal-treated group received 200 mg T, weekly for two reasons: (a) the pharmacokinetics of this

Table 1

Correlations among the independent variables (age, T, and impulsivity) and total aggression scores for all groups (All), eugonadal group (Eug: treated and placebo), and the hypogonadal group (hypo)

| Variables | Week 0 | | | Week 4 | | | Week 8 | | |
|-------------------------|--------|-------|------|--------|-------|------|--------|-------|------|
| | All | Eug | Hypo | All | Eug | Hypo | All | Eug | Hypo |
| Age | -.04 | -.16 | .00 | .10 | .01 | -.01 | -.08 | -.22 | -.10 |
| T | -.41* | -.21 | .48 | -.12 | .15 | .09 | -.15 | .02 | -.33 |
| Nonplanning impulsivity | .05 | .18 | .57 | -.15 | .00 | .49 | .02 | .21 | .65 |
| Cognitive impulsivity | .60** | .63** | .39 | .53** | .57** | .30 | .65** | .73** | .22 |
| Motor impulsivity | .56** | .48* | .56 | .56** | .54** | .30 | .63** | .56** | .59 |

* $P < .05$.

** $P < .01$.

T preparation are well-documented [23] and (b) this dosage has been used successfully in male contraceptive clinical trials [23]. One participant from the eugonadal-treated group had to withdraw from the study before Week 4 for personal reasons unrelated to the study. A wash-out period of 6–8 weeks was required for the hypogonadal group (to allow endogenous T levels to fall to the low hypogonadal range < 6 nmol/l, the normal range being 10–35 nmol/l) before they were admitted to the study. The hypogonadal group received 200 mg im T enanthate biweekly for 8 weeks. No significant age differences were found between the groups [$F(2,35) = 0.97$, ns]. All participants provided written consent. The study was approved by the Central Manchester Research Ethics Committee for Medical Research.

Volunteers completed a battery of validated behavioral scales (see below) at baseline (Week 0) and at Weeks 4 and 8. Mood was assessed weekly. The volunteers completed all the behavioral measures in the privacy of a clinical research room. Blood sampling was performed before receiving intramuscular injection at Weeks 0, 4, and 8. Time of venepuncture was random among the groups, although, generally each participant provided their sample at approximately the same time of day at each of the time points. All plasma samples were stored at -20°C until assay. T was measured using a time-resolved fluoroimmunoassay (AutoDELFIA Testosterone Kit) with an assay sensitivity of 0.4 nmol/l.

2.3. Instruments

The Barratt Impulsivity Scale-11 (BIS-11) [24] was administered at baseline to assess trait impulsivity. There are three subscales: nonplanning impulsivity, cognitive impulsivity, and motor impulsivity.

The AQ [25] was administered to assess aggression levels. There are four subscales: physical aggression, verbal aggression, anger, and hostility. A total aggression score was also calculated by summing the four subscales (and is the main dependent variable in Tables 1 and 2). For the purpose of this prospective study, the AQ, which is general in its referents, and can be characterised as a trait measure, was modified slightly to refer to a specific period in the

recent past (i.e. the last 4 weeks). This made the measure more suitable for measuring within-subject variation.

The Partner Aggression Questionnaire (AQ-P) is an adapted version of the AQ [25,26] where the male respondent's partner is asked to rate their partner in relation to each of the AQ items. Since the hypogonadal patients were generally not involved in long-term sexual relationships, no partner data were collected.

The Aggressive Provocation Questionnaire (APQ) [26] is a measure of aggressive responding. It consists of 21 vignettes that are representations of real provocative situations. The respondent is asked to imagine at this moment: (1) how he would feel in each situation (angry, frustrated, and irritated), measured on a five-point Likert scale and, (2) how he would react to each situation by choosing one of five action alternatives categorised as follows: (1) avoid, (2) no response, (3) anger, (4) assertive behavior, and (5) direct aggression. In the present study, analysis concentrated on the total number of aggressive and assertive options chosen across the 21 vignettes.

The State Self-Esteem Scale [27] consists of 20 items that are concerned with performance, social, and appearance aspects of self-esteem.

The Rathus Assertiveness Schedule [28] consists of 30 items, which are concerned with assessing to what extent the participant is assertive in a range of situations. Again for the purpose of this prospective study, the respondents were

Table 2

Stepwise multiple regression analysis: predicting total aggression scores

| Variable | Predictors | <i>t</i> | β | R^2 | <i>P</i> |
|---------------------------|--------------------------|----------|---------|-------|----------|
| Total aggression (Week 0) | 1. Cognitive impulsivity | 4.11 | .63 | .39 | $< .01$ |
| Total aggression (Week 4) | 1. Cognitive impulsivity | 3.53 | .57 | .32 | $< .01$ |
| | 1. Cognitive impulsivity | 2.44 | .41 | | $< .05$ |
| | 2. Motor impulsivity | 2.09 | .35 | .43 | $< .05$ |
| Change in $R^2 = .11$ | | | | | |
| Total aggression (Week 8) | 1. Cognitive impulsivity | 5.51 | .73 | .54 | $< .01$ |
| | 1. Cognitive impulsivity | 4.32 | .60 | | $< .01$ |
| | 2. Motor impulsivity | 2.11 | .29 | .61 | $< .05$ |
| Change in $R^2 = .07$ | | | | | |

instructed to rate themselves over a specific period in the recent past (i.e. last 4 weeks).

The irritability subscale from the BDHI [29] was adapted to be completed along a five-point scale and respondents were instructed to rate themselves over a specific period in the recent past (i.e. last 4 weeks).

The Profile of Mood States (POMS) [30] consists of 65 adjectives that describe feelings and mood. There are six subscales: tension–anxiety, depression–dejection, anger–hostility, vigor–activity, fatigue–inertia, and confusion–bewilderment. A total mood disturbance score was also calculated by subtracting the vigor–activity subscale score from the total summation of all other scales. The analysis concentrated on the mean score for each 2-week period (e.g. mean for Weeks 1 and 2).

2.4. Data analysis

Descriptive statistics were calculated for each of the variables. Preliminary Pearson's Product–Moment Correlational analysis and stepwise multiple regression were used to investigate the predictive relationship between the independent variables (e.g. T level, age, cognitive, motor, and nonplanning impulsivity scores) and the main dependent variable, total aggression (total aggression score includes the summation of each of the scored subscales from the AQ) at each time point. Two-factor analysis of variance for a mixed design was used to assess performance differences across testing sessions (time factor: Weeks 0, 4, and 8) and between groups (group factor: eugonadal-treated, eugonadal-placebo, and hypogonadal groups) for all dependent variables. Multiple comparisons were also made using Scheffe post hoc tests. All data were analysed using SPSS for Windows Version 9.0.

3. Results

3.1. Testosterone levels

T levels are shown in Fig. 1. Two-factor ANOVA revealed significant main effects for time [$F(2,68)=52.96$, $P<.01$] and group [$F(2,34)=38.99$, $P<.01$], and a significant Time \times Group interaction [$F(4,68)=16.39$, $P<.01$]. Post hoc analyses showed that exogenous T significantly increased T levels from a baseline level of 21.7 ± 1.6 – 34.4 ± 1.7 nmol/l at Week 4 to 38.4 ± 2.1 nmol/l at Week 8 in the eugonadal-treated group. In contrast, T levels of the eugonadal-placebo group did not change from a baseline level of 20.1 ± 1.4 nmol/l (Weeks 4 and 8 = 18.9 ± 1.3 and 20.0 ± 1.8 nmol/l, respectively). The hypogonadal group responded to T treatment with a significant increase into the normal range from a baseline level of 3.8 ± 1.1 – 18.1 ± 1.6 nmol/l at Week 4 to 22.4 ± 2.3 nmol/l at Week 8.

3.2. Trait impulsivity

One-way ANOVA revealed a significant difference between the groups for nonplanning impulsivity [$F(2,35)=2.66$, $P<.05$]. The hypogonadal group reported significantly lower levels of nonplanning impulsivity than the eugonadal-placebo group. Neither cognitive [$F(2,35)=0.89$, ns] or motor [$F(2,35)=1.95$, ns] impulsivity showed significant differences.

3.3. Preliminary correlational analysis

Table 1 shows the correlations between the independent variables (age, T level, and impulsivity) and the main dependent variable, total aggression at Weeks 0, 4, and 8.

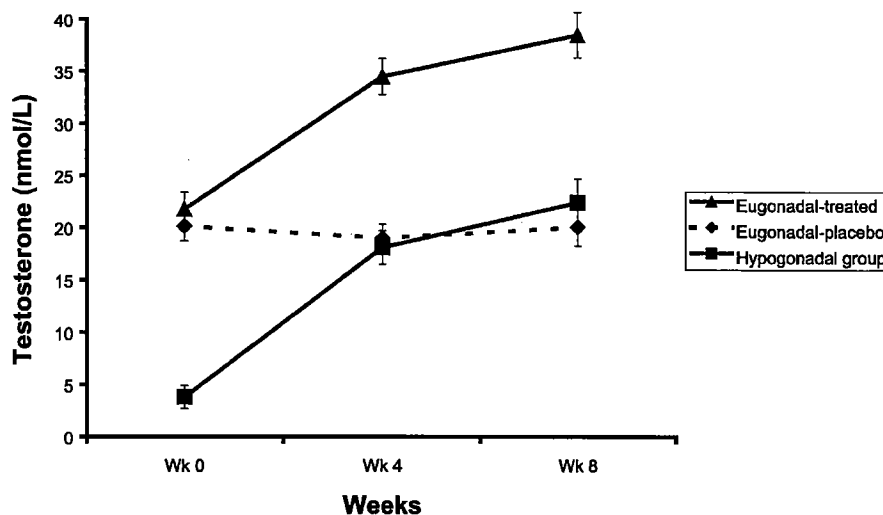


Fig. 1. Testosterone levels in all groups at baseline (week 0), week 4 and week 8.

All groups were first entered into the analysis, to provide greater variability in T levels. The analysis was rerun excluding the hypogonadal group.

At Week 0, only three significant correlations were found. Total aggression was negatively associated with T levels ($r = -.41$, $P < .05$) and positively associated with cognitive and motor impulsivity ($r = .60$, $P < .01$; $r = .56$, $P < .01$; respectively). When the hypogonadal group was excluded, only cognitive and motor impulsivity remained significantly correlated with total aggression levels ($r = .63$, $P < .01$; $r = .48$, $P < .05$; respectively). This indicates that higher levels of trait impulsivity are associated with higher levels of self-reported aggression in the eugonadal men.

At Week 4, two significant correlations were found for all participants combined. Again higher cognitive and motor impulsivity were significantly associated with higher levels of self-reported aggression ($r = .53$, $P < .01$; $r = .56$, $P < .01$; respectively). The correlations were similar after excluding the hypogonadal ($r = .57$, $P < .01$; $r = .54$, $P < .05$; respectively). At Week 8, cognitive and motor impulsivity were the only variables to be significantly associated with total aggression in the combined group and with the hypogonadal men omitted (Table 1).

3.4. Stepwise multiple regression analysis

Stepwise regression was used to further investigate the relationship between impulsivity and total aggression. Table 2 shows the results. At Week 0, only cognitive impulsivity emerged from the analysis, explaining 39% of the variance of total aggression scores. Cognitive impulsivity explained 32% of the variance in total aggression at Week 4, with motor impulsivity explaining an additional

11%. Similarly, at Week 8, cognitive impulsivity explained 54% of the variance in total aggression scores with motor impulsivity explaining a further 7%.

3.5. Aggression Questionnaire

Table 3 summarises self- and partner-reported scores on the AQ for each of the groups.

3.5.1. Physical aggression

No significant main effect was found for time [$F(2,68) = 0.61$, ns] or group [$F(2,34) = 1.29$, ns], suggesting that supra-physiological levels of T did not increase physical aggression. There was a slight trend towards a significant Time \times Group interaction [$F(4,68) = 2.05$, $P = .097$], which is likely to be accounted for by the increase in self-reported physical aggression in the hypogonadal group at Week 4. These findings were further endorsed by the partner reports in the eugonadal-treated and eugonadal-placebo groups. No significant main effects were found for time [$F(2,50) = 2.91$, ns], group [$F(1,25) = 0.80$, ns], or the Time \times Group interaction [$F(2,50) = 0.02$, ns].

3.5.2. Verbal aggression

A significant main effect was found for group [$F(2,34) = 3.39$, $P < .05$]. The hypogonadal group reported significantly higher levels of verbal aggression than the eugonadal-placebo group ($P < .05$). Neither main effect for time [$F(2,68) = 1.47$, ns] or the Time \times Group interaction [$F(4,68) = 0.49$, ns] were significant. Again, no significant main effects were found for partner reports, for time [$F(2,50) = 1.88$, ns], group [$F(1,25) = 0.65$, ns], or the Time \times Group interaction [$F(2,50) = 0.65$, ns].

Table 3

Descriptive statistics for self- and partner-reported scores on the aggression questionnaire at Weeks 0, 4, and 8

| Group | Self report | | | Partner report | | |
|----------------------------|--------------|--------------|--------------|----------------|--------------|--------------|
| | Week 0 | Week 4 | Week 8 | Week 0 | Week 4 | Week 8 |
| <i>Physical aggression</i> | | | | | | |
| Eugonadal-treated | 18.36 (1.61) | 19.07 (1.38) | 18.64 (1.77) | 16.29 (1.68) | 19.07 (1.38) | 17.00 (1.68) |
| Eugonadal-placebo | 18.20 (1.39) | 17.13 (1.22) | 18.06 (1.15) | 15.47 (1.13) | 17.13 (1.22) | 15.71 (1.32) |
| Hypogonadal | 19.75 (1.26) | 22.38 (2.03) | 19.75 (1.43) | | | |
| <i>Verbal aggression</i> | | | | | | |
| Eugonadal-treated | 14.07 (0.69) | 14.14 (0.60) | 13.79 (0.72) | 12.64 (1.10) | 12.23 (1.02) | 12.85 (1.05) |
| Eugonadal-placebo | 13.00 (0.74) | 13.66 (0.65) | 12.40 (0.56) | 11.27 (0.97) | 10.43 (1.05) | 10.64 (0.98) |
| Hypogonadal | 16.00 (1.53) | 16.38 (1.49) | 16.13 (1.69) | | | |
| <i>Hostility</i> | | | | | | |
| Eugonadal-treated | 17.11 (1.47) | 16.29 (1.23) | 15.21 (1.23) | 12.79 (1.09) | 13.69 (1.33) | 14.53 (1.34) |
| Eugonadal-placebo | 16.93 (1.08) | 16.00 (1.48) | 15.26 (1.10) | 12.53 (1.02) | 11.64 (0.80) | 11.21 (0.91) |
| Hypogonadal | 25.25 (2.18) | 26.88 (2.61) | 23.43 (2.38) | | | |
| <i>Anger</i> | | | | | | |
| Eugonadal-treated | 16.43 (1.26) | 16.14 (1.21) | 17.50 (0.74) | 14.57 (1.80) | 14.07 (1.72) | 16.46 (1.40) |
| Eugonadal-placebo | 14.93 (1.04) | 14.60 (1.13) | 15.13 (0.67) | 13.33 (1.38) | 12.79 (1.43) | 14.36 (1.12) |
| Hypogonadal | 20.00 (1.91) | 19.38 (1.38) | 19.50 (1.67) | | | |

Standard error of means are given in parentheses.

3.5.3. Hostility

A significant main effect was found for time [$F(2,68) = 4.89$, $P < .01$] and group [$F(2,34) = 11.11$, $P < .01$]. There was no significant Time \times Group interaction [$F(4,68) = 1.31$, ns]. The hypogonadal group reported significantly higher hostility scores than both the eugonadal-placebo and eugonadal-treated groups ($P < .01$). The significant main effect for time is accounted for by the reduced hostility scores in all groups by Weeks 4 and 8 (Week 4: 18.46 vs. Week 8: 17.27, $P < .05$; Week 0: 19.03 vs. Week 8: 17.27, $P < .01$). The partners generally reported lower levels of hostility than their counterparts. However, there were no significant main effects for time [$F(2,50) = 0.04$, ns], group [$F(1,25) = 2.07$, ns], or the Time \times Group interaction [$F(2,50) = 2.13$, ns].

3.5.4. Anger

No significant main effect was found for time [$F(2,68) = 1.96$, ns] or for the Time \times Group interaction [$F(4,68) = 0.83$, ns]. However, a significant main effect was found for group [$F(2,34) = 4.16$, $P < .05$]. The hypogonadal group reported significantly greater levels of anger compared to the eugonadal-placebo group ($P < .05$). For the partners' scores, a significant main effect was found for time [$F(2,50) = 4.69$, $P < .05$] but not for group [$F(1,25) = 2.07$, ns] or for the Time \times Group interaction [$F(2,50) = 0.20$, ns]. A significant increase in partner-reported anger scores from Weeks 4 to 8 accounted for this significant main effect ($t = 3.47$, $P < .01$).

3.6. Aggressive Provocation Questionnaire

3.6.1. Aggressive actions

No significant main effects were found for either time [$F(2,68) = 1.74$, ns] or group [$F(2,34) = 1.21$, ns]. There was a significant Time \times Group interaction [$F(4,68) = 2.70$, $P = .049$], but post hoc comparisons found no significant differences between any of the cells. These findings provide further support for an absence of a T treatment effect on levels of aggression (Table 4).

3.6.2. Assertive actions

There was a significant main effect for time [$F(2,68) = 4.02$, $P < .05$] but no significant effect for group [$F(2,34) = 0.92$, ns] or for the interaction [$F(4,68) = 0.98$, ns]. The main effect for time was explained by a significant increase in assertive actions from Weeks 0 to 4 (11.95 vs. 13.16; $t = 2.71$, $P < .01$). This trend was sustained at Week 8, although it was nonsignificant ($P = .07$), suggesting that the time effect may be accounted for by an overall placebo effect (Table 4).

3.6.3. Irritability

A significant main effect was found for group [$F(2,34) = 10.04$, $P < .01$], but not for time [$F(2,68) = 2.07$, ns] or for the Time \times Group interaction [$F(4,68) = 0.29$, ns]. Both the

Table 4

Descriptive statistics for behavior measures (on APQ) at Weeks 0, 4, and 8

| Group | Week 0 | Week 4 | Week 8 |
|---------------------------|--------------|--------------|--------------|
| <i>Aggressive actions</i> | | | |
| Eugonadal-treated | 0.93 (0.32) | 1.5 (0.54) | 0.86 (0.34) |
| Eugonadal-placebo | 0.67 (0.33) | 0.40 (0.19) | 1.00 (0.35) |
| Hypogonadal | 1.13 (0.61) | 1.88 (0.67) | 1.62 (0.46) |
| <i>Assertive actions</i> | | | |
| Eugonadal-treated | 11.86 (0.71) | 12.21 (0.80) | 12.71 (0.99) |
| Eugonadal-placebo | 11.47 (0.96) | 13.27 (1.30) | 12.07 (1.11) |
| Hypogonadal | 13.00 (1.12) | 14.63 (1.46) | 14.75 (1.18) |
| <i>Irritability</i> | | | |
| Eugonadal-treated | 28.29 (1.56) | 26.50 (1.51) | 25.64 (1.79) |
| Eugonadal-placebo | 23.87 (1.14) | 24.13 (1.40) | 23.00 (1.28) |
| Hypogonadal | 33.75 (2.40) | 33.75 (1.87) | 32.62 (1.76) |
| <i>State self-esteem</i> | | | |
| Eugonadal-treated | 51.21 (1.54) | 50.29 (1.88) | 51.29 (2.26) |
| Eugonadal-placebo | 48.60 (1.52) | 47.67 (1.32) | 48.53 (1.70) |
| Hypogonadal | 53.00 (2.57) | 53.25 (2.06) | 58.37 (1.88) |
| <i>Assertiveness</i> | | | |
| Eugonadal-treated | 10.21 (2.99) | 19.50 (5.40) | 19.29 (5.13) |
| Eugonadal-placebo | 15.67 (4.31) | 16.73 (4.60) | 19.27 (4.61) |
| Hypogonadal | 4.25 (5.79) | 8.87 (6.04) | 9.00 (5.07) |

Standard error of means are given in parentheses.

eugonadal-treated and eugonadal-placebo groups reported significantly lower levels of irritability overall than the hypogonadal group (Table 4).

3.6.4. State self-esteem

A significant main effect was found for both time [$F(2,68) = 4.86$, $P < .05$] and group [$F(2,34) = 3.28$, $P < .05$], with the Time \times Group interaction approaching significance [$F(4,68) = 2.39$, $P = .059$]. The significant main effect for time is accounted for by the increase in self-esteem scores from 49.86 at Week 4 to 51.70 at Week 8 ($t = 2.13$, $P < .05$), in particular by the increase reported by the hypogonadal group from Weeks 4 to 8 (53.25 vs. 58.37). This finding (Table 4) reflects the trend towards a significant interaction, suggesting the existence of a positive treatment effect on self-esteem in the hypogonadal group.

3.6.5. Assertiveness

A significant main effect was found for time [$F(2,68) = 6.44$, $P < .01$] but not for group [$F(4,68) = 1.24$, ns] or their interaction [$F(2,34) = 1.09$, ns]. Table 4 shows substantial increases in assertiveness at Week 4 compared with Week 0, particularly in the eugonadal-treated and hypogonadal groups.

3.7. Profiles of Mood States

3.7.1. Total mood disturbance

Significant main effects were found for time [$F(4,136) = 6.72$, $P < .01$], for group [$F(2,34) = 13.04$, $P < .01$], and for

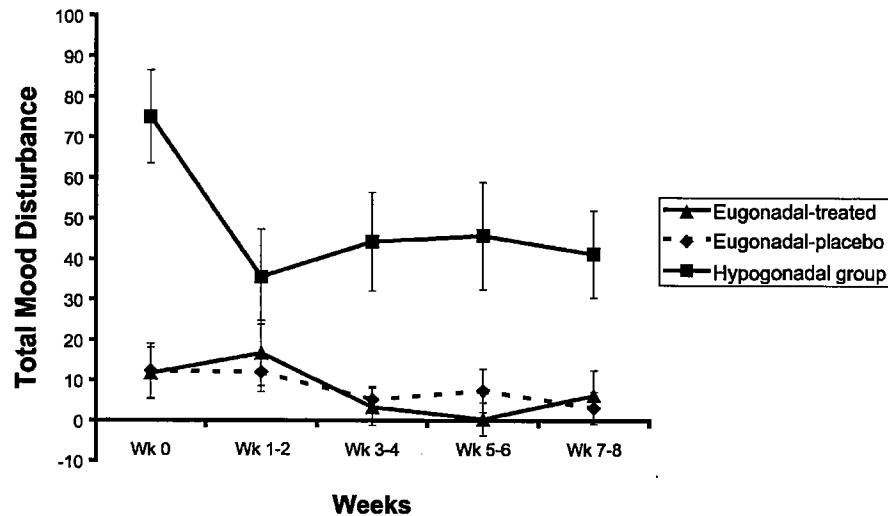


Fig. 2. Total mood disturbance in all groups at baseline (week 0), week 4 and week 8.

the Time \times Group interaction [$F(8,136) = 3.320$, $P < .01$]. Multiple comparisons found that the significant group effect was accounted for by significantly higher levels of total mood disturbance in the hypogonadal group than the eugonadal-treated and eugonadal-placebo groups (see Fig. 2). However, there was a significant reduction in total mood

scores in the hypogonadal group by Weeks 1–2 explaining the significant interaction effect ($t = 3.07$, $P < .05$).

3.7.2. POMS subscales

POMS subscale scores are shown in Table 5. Significant main effects were found for time for tension–anxiety

Table 5
Descriptive statistics for subscales on POMS at Weeks 0, 1–2, 3–4, 5–6, and 7–8

| Group | Week 0 | Week 1–2 | Week 3–4 | Week 5–6 | Week 7–8 |
|-------------------------------|--------------|--------------|--------------|--------------|--------------|
| <i>Tension–anxiety</i> | | | | | |
| Eugonadal-treated | 6.64 (1.09) | 7.71 (1.45) | 5.71 (0.99) | 5.21 (1.10) | 6.68 (1.35) |
| Eugonadal-placebo | 6.40 (1.16) | 5.80 (0.98) | 4.90 (0.76) | 5.30 (0.89) | 4.77 (0.79) |
| Hypogonadal | 17.37 (1.84) | 9.75 (1.12) | 11.63 (1.72) | 11.38 (1.89) | 12.00 (2.01) |
| <i>Depression–dejection</i> | | | | | |
| Eugonadal-treated | 5.07 (1.29) | 5.93 (1.45) | 2.93 (1.20) | 1.68 (0.50) | 1.82 (0.65) |
| Eugonadal-placebo | 5.73 (2.05) | 4.37 (1.34) | 2.70 (0.85) | 3.60 (1.67) | 2.23 (1.20) |
| Hypogonadal | 20.13 (3.83) | 11.25 (3.55) | 12.06 (4.03) | 12.69 (4.08) | 11.44 (3.32) |
| <i>Anger–hostility</i> | | | | | |
| Eugonadal-treated | 7.29 (1.88) | 9.11 (2.20) | 4.86 (1.28) | 3.46 (0.83) | 5.54 (1.79) |
| Eugonadal-placebo | 3.73 (1.33) | 4.03 (0.82) | 4.10 (0.82) | 4.00 (0.99) | 3.13 (0.67) |
| Hypogonadal | 17.75 (2.37) | 12.06 (2.90) | 17.69 (2.70) | 16.88 (2.85) | 14.75 (1.95) |
| <i>Vigor–activity</i> | | | | | |
| Eugonadal-treated | 20.79 (1.63) | 18.36 (1.70) | 18.43 (1.71) | 17.89 (1.87) | 17.79 (2.00) |
| Eugonadal-placebo | 17.06 (1.41) | 16.50 (1.46) | 17.87 (1.24) | 16.47 (0.98) | 16.87 (1.04) |
| Hypogonadal | 10.63 (2.26) | 15.88 (2.22) | 17.06 (1.80) | 14.75 (1.71) | 17.50 (1.75) |
| <i>Fatigue–inertia</i> | | | | | |
| Eugonadal-treated | 7.36 (1.44) | 7.43 (1.25) | 4.25 (0.71) | 4.32 (0.76) | 6.50 (1.52) |
| Eugonadal-placebo | 6.47 (1.01) | 8.40 (1.05) | 6.30 (0.84) | 6.13 (1.21) | 5.30 (0.87) |
| Hypogonadal | 18.12 (2.91) | 9.56 (2.29) | 11.19 (2.76) | 10.81 (2.92) | 12.06 (2.48) |
| <i>Confusion–bewilderment</i> | | | | | |
| Eugonadal-treated | 6.14 (0.97) | 5.32 (1.27) | 4.04 (0.95) | 3.57 (0.94) | 3.50 (0.87) |
| Eugonadal-placebo | 7.07 (1.11) | 5.73 (1.01) | 5.03 (0.78) | 4.87 (0.98) | 4.60 (0.98) |
| Hypogonadal | 12.25 (1.45) | 8.81 (1.16) | 8.75 (1.11) | 8.56 (1.61) | 8.44 (1.15) |

Standard error of means are given in parentheses.

[$F(4,136)=6.80$, $P<.01$], for depression–dejection [$F(4,136)=7.81$, $P<.01$], for fatigue–inertia [$F(4,136)=4.84$, $P<.01$], for confusion–bewilderment [$F(4,136)=11.96$, $P<.01$] but not for anger–hostility [$F(4,136)=0.79$, ns] or vigor–activity [$F(4,136)=1.54$, ns]. Significant main effects were found for group for tension–anxiety [$F(2,34)=10.83$, $P<.01$], for depression–dejection [$F(2,34)=10.70$, $P<.01$], for anger–hostility [$F(2,34)=27.03$, $P<.01$], for fatigue–inertia [$F(2,34)=8.49$, $P<.01$], for confusion–bewilderment [$F(2,34)=5.80$, $P<.01$] but not for vigor–activity [$F(2,34)=1.28$, ns]. The hypogonadal group reported significantly higher levels of tension–anxiety, depression–dejection, anger–hostility, fatigue–inertia, and confusion–bewilderment than the eugonadal men ($P<.01$). There was a significant Time \times Group interaction for tension–anxiety interaction [$F(8,136)=4.02$, $P<.01$], for anger–hostility [$F(8,136)=2.36$, $P<.05$], for vigor–activity [$F(8,136)=3.84$, $P<.01$], and for fatigue–inertia [$F(8,136)=3.02$, $P<.01$]. The Time \times Group interaction for depression–dejection [$F(8,136)=1.97$, $P=.08$] and for confusion–bewilderment [$F(8,136)=0.61$, ns] was not significant. Planned comparisons showed that significant reduction in tension–anxiety, anger–hostility, and fatigue–inertia by Weeks 1–2 and an increase in vigor–activity by Weeks 3–4 in the hypogonadal group explained the interaction effects, indicating a T treatment effect.

4. Discussion

We found that (trait) cognitive impulsivity (i.e. the tendency to make up one's mind quickly) and (trait) motor impulsivity (i.e. the tendency to act on impulse) accounted for significant amounts of the variance in total aggression levels, over and above age and level of T. This highlights the importance of including individual difference explanatory variables in experimental studies in this area. This finding adds support to an earlier study of violent and nonviolent male parolees by Cherek et al. [31]. They found that violent parolees scored significantly higher on a 'delayed gratification' impulsivity laboratory measure and on the BIS-11 compared to the nonviolent parolees. Furthermore, in another report using the same sample, Cherek et al. [32] found the number of impulsive choices chosen in all parolees to be significantly correlated with the number of aggressive responses reported previously.

Similarly, Galligani et al. [3] found in a study comparing AAS users with a drug-free group that the former reported levels of impulsiveness at least one standard deviation above the mean. The AAS users also reported significantly greater levels of indirect and verbal aggression. Another very recent study found impulsivity to predict AAS using group. That is, young men who report AAS use score significantly higher than non-AAS users [33] on impulsivity and self-reported aggression. Such data, taken as a whole suggest that an underlying mechanism, which inhibits

aggressive behavior, may be less effective, thus contributing to the aetiology of aggressive behavior in normal men. In other words, the link between steroid use and aggression may be that impulsive men are more likely to take steroids and also be aggressive.

However, given the methodological limitations of previous studies (i.e. overreliance of cross-sectional designs), it is difficult to decipher the exact nature of the relationship between impulsivity and aggressive behavior. Deficits in serotonin levels have been implicated in the development of both impulsive and aggressive behavior [34,35]. One recent study [35] found a significant reduction in impulsive responses in a sample of adult males with a history of conduct disorder after administration of D,L-fenfluramine—a drug which releases serotonin (and dopamine). Another found that laboratory aggressive responding increased following the ingestion of a tryptophan (a serotonin precursor)-depleting beverage supporting the notion that serotonin has a role in regulating aggression [36].

We also found that exogenous T administration did not lead to significant increases in a range of aggression measures in eugonadal men. This is not congruent with other experimental studies [15,16]. Most recently, Pope et al. [15] reported a significant increase in both aggressive responding as measured by the PSAP and ratings of manic symptoms. They suggested that the AQ may not be as sensitive as other aggression measures (cf. Ref. [37]) and that the Young Mania Rating Scale [38] and the PSAP were better adapted to detect T effects. This is a moot point given that an earlier paper by the authors [20] reported detecting T treatment effects using the AQ. Moreover, one would have predicted in the present study that our measure of irritability might have at the very least detected any real manic-like related changes. However, it is worth noting that when effect sizes were calculated for Weeks 0–4 comparisons, there were small effects in the direction of an increase in physical aggression. Although, nonsignificant these would need to be borne in mind for future meta-analyses since a series of nonsignificant findings that are all in the same direction may add up to a weak but significant effect when such studies are combined.

It is also reasonable to speculate that the subtle but significant adverse psychological effects reported elsewhere in the literature [16] are only associated with doses of T substantially higher than that employed in this study (>200 mg/week) or utilised in male contraception clinical trials. If this is the case, further high-dose studies are required using detailed self- and partner-reported behavioral and mood assessments in a double-blind, placebo-controlled, cross-over fashion.

We also failed to find any T-related mood effects in the eugonadal-treated group. However, the hypogonadals were identified as the group who benefited most from the T treatment. Significant reductions in tension, anger, and fatigue were reported by Weeks 1–2, followed by a significant increase in vigor by Weeks 3–4. There was also a trend towards a significant Time \times Group interaction for

state self-esteem levels, indicating a marked increase in levels of self-esteem in the hypogonadal group. These findings support earlier research that has found T administration to have positive mood effects when T levels are restored into the normal range [22,39] and suggest that prolonged treatment is likely to maintain these mood benefits, but not further improve them [40]. It is likely the marked elevations in self-esteem scores and positive mood states are concomitant with the restoration of normal sexual function as reported elsewhere [40]. Therefore, it is possible the changes in the psychological functioning are related to changes in sexual function.

The hypogonadal group generally reported significantly elevated levels of verbal aggression, hostility, anger, and irritability compared to both the eugonadal groups. We recognize that a hypogonadal–placebo group has not been included in the design of our study. Ethically and clinically, it would be problematic to treat hypogonadal men with a placebo in order to investigate behavioral changes. However, despite this, it is important to note that the ‘nonblinded’ hypogonadal group did not consistently report lower levels of aggression, hostility, anger, or irritability (comparable to both the eugonadal groups) after treatment commenced. Instead, their self-reported levels remained significantly higher, possibly indicating real concerns as opposed to a placebo effect. To the best of our knowledge, this is the first study to demonstrate the extent of the negative affect experienced by this patient population. These data have implications clinically, for both treatment and therapy.

In conclusion, we have found that supraphysiological levels of T do not lead to significantly increased aggression or mood disturbances. Instead, the inability to control one's behavior when such control is required by a particular situation was found to significantly predict levels of aggression over and above age and T level. Finally, our data for the first time have identified an unacceptably high level of negative affect experienced by hypogonadal patients before and during treatment that requires further investigation.

Acknowledgments

We are grateful to the World Health Organisation (Program No. 96374 to Wu and Archer) for funding this study, to Dr. Helen Buckler and Sister Marie Vickers (Hope Hospital, Manchester, UK) for assisting in the recruitment of hypogonadal patients, and to the Radioimmunoassay Laboratory personnel (Central Manchester Healthcare NHS Trust, UK) for T measurement.

References

- [1] Tricker R, Casaburi R, Storer TW, Clevenger B, Berman N, Shirazi A, Bhasin S. The effect of supraphysiological doses of testosterone on angry behavior in healthy eugonadal men: a clinical research center study. *J Clin Endocrinol Metab* 1996;81:3754–8.
- [2] Parrott AC, Choi PYL, Davies M. Anabolic-steroid use by amateur athletes — effects upon psychological mood states. *J Sports Med Phys Fitness* 1994;34:292–8.
- [3] Galligani N, Renck A, Hansen S. Personality profile of men using anabolic–androgenic steroids. *Horm Behav* 1996;30:170–5.
- [4] Sharp M, Collins D. Exploring the “inevitability” of the relationship between anabolic–androgenic steroid use and aggression in human males. *J Sport Exercise Psychol* 1998;20:379–94.
- [5] Choi PYL, Pope HG. Violence toward women and illicit androgenic–anabolic steroids. *Ann Clin Psychiatry* 1994;6:21–5.
- [6] World Health Organisation Task Force on Methods for the Regulation of Male Fertility. Contraceptive efficacy of testosterone-induced azoospermia in normal men. *Lancet* 1990;336:955–9.
- [7] Rabkin JG, Rabkin R, Wagner G. Testosterone replacement therapy in HIV illness. *Gen Hosp Psychiatry* 1995;17:37–42.
- [8] Tenover JS. Androgen administration to aging men. *Endocrinol Metab Clin North Am* 1994;23:877–92.
- [9] Archer J. The influence of testosterone on human aggression. *Br J Psychol* 1991;82:1–28.
- [10] Aromaki AS, Lindman RE, Eriksson CJP. Testosterone, aggressiveness and antisocial personality. *Aggressive Behav* 1999;25:113–23.
- [11] Virkkunen M, Kallio E, Rawlings R, Tokola R, Poland RE, Guidotti A, Nemeroff C, Bissette G, Kalogeras K, Karonen S, Linnoila M. Personality profiles and state aggressiveness in Finnish alcoholic, violent offenders, fire setters, and healthy volunteers. *Arch Gen Psychiatry* 1994;51:28–33.
- [12] Archer J, Biring SS, Wu FCW. The association between testosterone and aggression among young men—empirical findings and a meta-analysis. *Aggressive Behav* 1998;24:411–20.
- [13] Dabbs JM, Jurkovic GL, Frady RL. Salivary testosterone and cortisol levels among late adolescent male offenders. *J Abnorm Child Psychol* 1991;19:469–78.
- [14] Anderson RA, Bancroft J, Wu FCW. The effects of exogenous testosterone on sexuality and mood of normal men. *J Clin Endocrinol Metab* 1992;75:1503–7.
- [15] Pope HG, Kouri EM, Hudson JI. Effects of supraphysiologic doses of testosterone on mood and aggression in normal men: a randomised controlled trial. *Arch Gen Psychiatry* 2000;57:133–40.
- [16] Su T-P, Pagliaro M, Schmidt PJ, Pickar D, Wolkowitz O, Rubinow DR. Neuropsychiatric effects of anabolic steroids in male normal volunteers. *JAMA, J Am Med Assoc* 1993;269:2760–4.
- [17] Yates WR, Perry PJ, MacIndoe J, Holman T, Ellingrod V. Psychosexual effects of three doses of testosterone cycling in normal men. *Biol Psychiatry* 1999;45:254–60.
- [18] Johnson BT. Software for the meta-analytic review of research literatures. Hillsdale (NJ): Erlbaum, 1989.
- [19] Cherek DR, Schnapp W, Moeller FG, Dougherty DM. Laboratory measures of aggressive responding in male parolees with violent and nonviolent histories. *Aggressive Behav* 1996;22:27–36.
- [20] Kouri EM, Lukas SE, Pope HG, Oliva PS. Increased aggressive responding in male volunteers following the administration of gradually increasing doses of testosterone cypionate. *Drug Alcohol Depend* 1995;40:73–9.
- [21] Bjorkqvist K, Nygren T, Bjorklund A, Bjorkqvist S. Testosterone intake and aggressiveness: real effect or anticipation? *Aggressive Behav* 1994;20:17–26.
- [22] Alexander GM, Swerdloff RS, Wang C, Davidson T, McDonald V, Steiner B, Hines M. Androgen–behaviour correlations in hypogonadal men and eugonadal men. *Horm Behav* 1998;33:85–94.
- [23] Anderson RA, Wu FCW. Comparison between testosterone enanthate-induced azoospermia and oligozoospermia in a male contraceptive study: II. Pharmacokinetics and pharmacodynamics of once weekly administration of testosterone enanthate. *J Clin Endocrinol Metab* 1996;81:896–901.
- [24] Barratt ES. Impulsiveness defined within a systems model of person-

- ality. In: Spielberger ED, Butcher JN, editors. *Advances in personality assessment*, vol. 5. Hillsdale (NJ): Erlbaum, 1985, pp. 13–45.
- [25] Buss AH, Perry M. The Aggression Questionnaire. *J Pers Soc Psychol* 1992;63:452–9.
- [26] O'Connor DB, Archer J, Wu FCW. Measuring aggression: self-reports, partner reports and responses to provoking scenarios. *Aggressive Behav* 2001;27:79–101.
- [27] Heatherton TF, Polivy J. Development and validation of a scale for measuring state self-esteem. *J Pers Soc Psychol* 1991;60:895–910.
- [28] Rathus SA. A 30-item schedule for assessing assertive behavior. *Behav Ther* 1973;4:398–406.
- [29] Buss AH, Durkee A. An inventory for assessing different kinds of hostility. *J Consult Psychol* 1957;21:343–9.
- [30] McNair DM, Lorr M, Droppleman LF. *EdITS manual for the Profile of Mood States*. San Diego (CA): Educational and Industrial Testing Service, 1992.
- [31] Cherek DR, Moeller F, Dougherty DM, Rhoades H. Studies of violent and nonviolent male parolees: II. Laboratory and psychometric measurements of impulsivity. *Biol Psychiatry* 1997;41:523–9.
- [32] Cherek DR, Moeller F, Schnapp W, Dougherty DM. Studies of violent and nonviolent male parolees: I. Laboratory and psychometric measurements of aggression. *Biol Psychiatry* 1997;41:514–22.
- [33] Field C. Personality, aggressive behaviour and anabolic-androgenic steroid use in a sample of men. Unpublished BSc dissertation, University of Leeds, School of Psychology, Leeds, UK, 2001.
- [34] Coccaro EF, Berman ME, Kavoussi RJ, Hauger RL. Relationship of prolactin response to D-fenfluramine to behavioral and questionnaire assessments of aggression in personality disordered men. *Biol Psychiatry* 1996;40:157–64.
- [35] Cherek DR, Lane SD. Fenfluramine effects on impulsivity in a sample of adults with and without history of conduct disorder. *Psychopharmacology* 2000;152:149–56.
- [36] Bjork JM, Dougherty DM, Moeller FG, Cherek DR, Swann AC. The effects of tryptophan depletion and loading on laboratory aggression in men: time course and a food restricted control. *Psychopharmacology* 1999;142:24–30.
- [37] Allen TJ, Dougherty DM, Rhoades HM, Cherek DR. A study of male and female aggressive responding under conditions providing an escape response. *Psychol Rec* 1996;46:651–64.
- [38] Young RC, Biggs JT, Ziegler VE, Meyer DA. A rating scale for mania: reliability, validity and sensitivity. *Br J Psychiatry* 1978;133:429–35.
- [39] Anderson RA, Martin CW, Kung AWC, Everington D, Pun TC, Tan KCB, Bancroft J, Sundaram K, Moo-Young AJ, Baird DT. 7 α -Methyl-19-Nortestosterone maintains sexual behaviour and mood in hypogonadal men. *J Clin Endocrinol Metab* 1999;84:3556–62.
- [40] Wang C, Alexander G, Berman N, Salehian B, Davidson T, McDonald V, Steiner B, Hull L, Callegari C, Swerdloff RS. Testosterone replacement therapy improves mood in hypogonadal men—a clinical research center study. *J Clin Endocrinol Metab* 1996;81:3578–83.

AA2500 Testosterone Gel Normalizes Androgen Levels in Aging Males with Improvements in Body Composition and Sexual Function

C. STEIDLE, S. SCHWARTZ, K. JACOBY, T. SEBREE, T. SMITH, R. BACHAND, AND THE NORTH AMERICAN AA2500 T GEL STUDY GROUP

Northeast Indiana Research (C.S.), Fort Wayne, Indiana 46825; Diabetes and Glandular Disease Research Associates (S.S.), San Antonio, Texas 78229; Integrity Medical Research (K.J.), Seattle, Washington 98133; and Auxilium Pharmaceuticals, Inc. (T.Se., T.Sm., R.B.), Norristown, Pennsylvania 19041

Testosterone replacement in hypogonadal men improves body composition, mood, and sexual functioning. In this 90-d study, we compared the pharmacokinetics and treatment effectiveness of a topical testosterone gel (AA2500) at two concentrations, 50 mg/d and 100 mg/d, to a testosterone patch and placebo gel in 406 hypogonadal men. Pharmacokinetic profiles were obtained, body composition was measured, and mood and sexual function were monitored. AA2500 treatments resulted in dose-dependent improvements in all pharmacokinetic parameters, compared with testosterone patch and placebo. Mean average concentrations at d 90 T were 13.8, 17.1, 11.9, and 7.3 nmol/liter for 50 mg/d AA2500, 100 mg/d AA2500,

testosterone patch, and placebo, respectively. At d 90, the 100 mg/d AA2500 treatment improved lean body mass by 1.7 kg and percentage of body fat by 1.2% to a significantly greater degree than either control treatment. Significant improvements in spontaneous erections, sexual desire, and sexual motivation were also evidenced with the 100 mg/d AA2500 dose in comparison with placebo. Testosterone gel was well tolerated; however, the testosterone patch resulted in a high rate of application site reactions. Overall, AA2500 is an effective, well tolerated treatment for hypogonadism. (*J Clin Endocrinol Metab* 88: 2673–2681, 2003)

THE USE OF testosterone (T) replacement therapy in hypogonadal men has been well documented. Specifically, restoration of serum T concentrations to within normal limits (*i.e.* similar to that of eugonadal men) can maintain sexual characteristics, sexual behavior, energy, mood, and muscle development and improve bone density (1). Currently there are a number of different T dosage forms available for replacement therapy in hypogonadal men, but many of these formulations have limitations. Orally available T is relatively insoluble and subject to a high first-pass effect in the liver. Intramuscular depot injections are used widely for replacement therapy but are inconvenient and result in wide fluctuations in T levels. Specifically, high initial peak levels, followed by serum T levels below the lower limit of normal toward the end of the cycle, lead to a return of clinical signs and symptoms.

The skin and oral mucosa are considered favorable routes for the delivery of T. Transdermal T patches, including the scrotal patch (Testoderm), the nonscrotal permeation-enhanced patch with an alcohol-based reservoir (Androderm), and the nonscrotal patch without a reservoir (Testoderm TTS) provide a more consistent delivery of T into the systemic circulation, although serum T levels are not always

maintained within normal limits over a 24-h period. Long-term use of these patches (3–10 yr) has been shown to be effective in maintaining sexual function and bone and muscle mass in both young and elderly hypogonadal males (2–5); however, skin tolerability problems or the need for shaving large areas of scrotal skin invariably affect compliance with transdermal patches. Skin reactions commonly occur at the patch application site, particularly with the permeation-enhanced T patches causing erythema or pruritus. Blister reactions also occur leading to scarring and discontinuation of treatment (6, 7).

Previously, it has been reported that a T gel (AndroGel), when applied over a larger area of skin, can achieve serum T levels in the normal range and produce less skin irritation than T patches. A new, unique topical T gel formulation (AA2500) has been designed to provide consistent transdermal absorption of T over 24 h after a single dose and is hereby reported. Before this study, the pharmacokinetic (PK) profile of this new T gel (AA2500) was compared with AndroGel. Data have demonstrated that after topical application of a single dose of AA2500 T gel or AndroGel, the time to maximum concentration (T_{max}) was comparable between the two formulations indicating no appreciable differences in the rate of absorption. However, the 0- to 24-h area under the curve (AUC_{0-24}) and maximum concentration (C_{max}) were consistently higher following application of AA2500 with approximately 30% higher serum T levels being noted. The safety profile of these two topical gel formulations was similar (8).

The study reported here involves comparisons among four parallel treatment groups in 406 patients consisting primarily of aging males with low serum T and associated signs and

Abbreviations: AUC_{0-24} , 0- to 24-h Area under the curve; BPH, benign prostatic hyperplasia; C_{avg} , mean concentration; C_{max} , maximum concentration; C_{min} , minimum concentration; DHT, dihydrotestosterone; DRE, digital rectal examination; %F, percentage fat; FM, fat mass; HDL, high-density lipoprotein; I-PSS, International Prostate Symptom Score; LBM, lean body mass; LDL, low-density lipoprotein; PK, pharmacokinetic; PSA, prostate-specific antigen; T, testosterone; TBM, total body mass; TC, total cholesterol.

symptoms of hypogonadism. Two doses of AA2500 T gel (50 mg/d and 100 mg/d) were compared with a T patch treatment (Androderm, two patches delivering 5 mg T daily), a dose that is known to give rise to clinically meaningful increases in serum T levels with amelioration of signs and symptoms (9). The fourth parallel group was a matching placebo gel to provide a blinded comparator for the two doses of AA2500 T gel, which also provided a valid overall assessment of clinical and subjective symptom improvement. In this 90-d study, periodic 24-h PK profiles of total T and dihydrotestosterone (DHT) were obtained, and the effect of normalizing serum T on body composition, sexual function, mood, and bone mineral density were assessed. Routine safety evaluations were conducted, including skin irritation assessments at the study drug application site. As such, this study design was robust and unique in the assessment of the efficacy of topical transdermal T in normalizing serum T levels, ameliorating signs and symptoms of hypogonadism, and assessing its safety.

Subjects and Methods

Subjects

Four hundred six male patients were randomized and treated at 43 clinics in the United States. Approximately 100 patients were randomized to each treatment group (Table 1). Patients were between 20 and 80 yr of age and had a morning T level of 10.4 nmol/liter or less at screening (measured at a central laboratory) and one or more symptoms of low T (*i.e.* fatigue, decreased muscle mass, reduced libido, reduced sexual functioning of a nonmechanical nature). Except for hypogonadism, the patients were in generally good health as evidenced by medical history; complete physical examination including a digital rectal examination (DRE), 12-lead electrocardiogram, vital sign assessments, clinical laboratory and urinalysis assessments, prostate assessment [International Prostate Symptom Score (I-PSS)]; and normal tests for prostate-specific antigen (PSA), hepatitis, and drugs of abuse. If the patient was receiving lipid-lowering agents, anxiolytics, lithium, antidepressants, hypnotics, antipsychotics, α_1 blockers, or herbal treatments for benign prostatic hyperplasia (BPH), the dose had to have been stable for at least 3 months before entering the study. Patients were excluded from the study if they had any generalized skin irritation or disease that might have interfered with androgen absorption; had received any estrogen therapy, an LHRH antagonist, human GH therapy; or had a history of drug abuse within

12 months. Also excluded were patients who had used either Viagra or apomorphine within 30 d or were treated with T or anabolic supplements within 6 wk before the study. The study was conducted in accordance with the Declaration of Helsinki and complied with Good Clinical Practice, and all patients signed an informed consent agreement previously approved by one of the participating institutional review boards.

Study drugs

AA2500 T gel (Testim) was supplied by Auxilium Pharmaceuticals, Inc. (Norristown, PA). The four daily treatments under study were 50 mg/d AA2500 or 100 mg/d AA2500, matching placebo gel, and a transdermal T patch (Androderm, two patches \times 2.5 mg T), each containing 12.2 mg T. The AA2500 and placebo gel were identical and applied as two tubes of 50 mg T (100 mg/d), one tube of 50 mg T and one tube of placebo (50 mg/d), or two tubes of placebo. Neither the patients nor the investigators were aware of the contents of the tubes.

All study drug treatments were applied in the morning; repeat applications occurred at the same time of day for the duration of the study. Each day in the gel-treated group, patients applied the contents of two tubes. The content of one tube was applied to one shoulder and the content of the remaining tube was applied to the other shoulder. Patients allocated to receive the T patch applied two adhesive patches daily. Application sites included the back, abdomen, upper arms, and thighs. Patches were to be worn for 24 h and then replaced each morning at approximately the same time.

Study design

The study was designed as a randomized, multidose, multicenter, active, and placebo-controlled study. Patients were randomized to 50 mg AA2500 T gel (99 patients) or 100 mg AA2500 T gel (106 patients), matching placebo gel (99 patients), or T patch (102 patients). Randomization was performed to ensure an equal distribution of treatments across study centers. The study was double blinded for the AA2500 and placebo groups and open label for the T patch group. Patients randomized to one of the two AA2500 arms could be titrated at d 60 based on their d 30 T PK profile. Patients were titrated from 50 mg/d to 100 mg/d at d 60 if their d 30 mean serum T concentration (C_{avg}) was less than 10.4 nmol/liter (300 ng/dl). Patients were titrated from 100 mg/d to 50 mg/d at d 60 if their d 30 C_{avg} was more than 34.7 nmol/liter (1000 ng/dl). These titration decisions were undertaken by a third-party physician who was unaware of any clinical aspects of the individual patients and not otherwise involved in the study.

On d -1, patients had a baseline 24-h profile for serum T and DHT consisting of serum samples taken at 0800, 1000, and 1200 h, and 1600

TABLE 1. Subject characteristics

| | AA2500 | | T patch | Placebo | Total |
|-------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | 50 mg/d | 100 mg/d | | | |
| Demographics | | | | | |
| n | 99 | 106 | 102 | 99 | 406 |
| Age (yr) | 58.1 \pm 9.7 | 56.8 \pm 10.6 | 60.5 \pm 9.7 | 56.8 \pm 10.8 | 58.0 \pm 10.3 |
| Height (cm) | 178 \pm 6 | 178 \pm 8 | 178 \pm 6 | 180 \pm 7 | 179 \pm 7 |
| Weight (kg) | 95.7 \pm 13.4 | 95.7 \pm 14.4 | 95.1 \pm 13.5 | 98.5 \pm 15.6 | 96.2 \pm 14.2 |
| BMI | 30.0 \pm 3.7 | 29.9 \pm 3.3 | 29.9 \pm 3.8 | 30.3 \pm 3.8 | 30.0 \pm 3.6 |
| T (nmol/liter) ^a | 8.1 \pm 2.0 | 8.1 \pm 2.2 | 8.3 \pm 2.4 | 7.9 \pm 2.8 | 8.1 \pm 2.3 |
| I-PSS score | 6.5 \pm 6.0 | 4.8 \pm 5.0 | 6.2 \pm 5.5 | 5.0 \pm 5.3 | 5.6 \pm 5.5 |
| PSA (ng/ml) | 1.17 \pm 0.89 | 1.29 \pm 0.96 | 1.45 \pm 1.18 | 1.13 \pm 1.00 | 1.26 \pm 1.02 |
| Cause of hypogonadism ^b | | | | | |
| Primary (n) | 8 | 7 | 4 | 3 | 22 |
| Secondary (n) | 91 | 98 | 98 | 95 | 382 |
| Aging (%) ^c | 70.7 | 58.1 | 66.7 | 61.2 | 64.1 |
| Normogonadotrophic (%) ^c | 19.2 | 30.5 | 26.5 | 31.6 | 27.0 |

Demographic values are expressed as means \pm 1 SD. BMI, Body mass index.

^a 0800 h serum concentration at screening examination.

^b Two subjects had a missing cause of hypogonadism.

^c Percentage of total by treatment group. Some subjects had more than one symptom, but all were required to have at least one. Distribution by cause is shown only if it occurred in $\geq 4\%$ of subjects.

and 0800 h on d 1, immediately before the first dose of study drug. On d 30 and 90, patients had a 24-h profile for T and DHT consisting of serum samples at predose and 2, 4, 8, 12, and 24 h after study drug administration. On d 60, a single 0800-h serum sample was taken for T and DHT. Blood samples for clinical laboratory assessment were collected at screening and on d -1, 30, 60, and 90. The prostate was evaluated at screening (PSA levels only) and d -1 and 90 with PSA levels, I-PSS, and DRE. Body composition [total body mass (TBM), lean body mass (LBM), and fat mass (FM)] and bone mineral density of the L1-L4 section of the lumbar spine were measured by dual energy x-ray absorptiometry on d -1 and d 90. Percentage fat (%F) was derived from FM and TBM. All body composition and bone mineral density measurements were centrally monitored and analyzed by Synarc, Inc. (Maynard, MA). Sexual function and mood questionnaires were recorded daily for 14 d before d 1 and daily for 7 d before d 30, 60, and 90. Data were collected centrally in real time via an interactive voice response system using the telephone. Skin irritation examination using a standardized, discrete scoring system was performed at d 1 (before dosing), 30, 60, and 90. Medical history and physical exams were completed, and all adverse events were recorded.

Methods

The skin irritation scoring was based on the following schema: 0, no erythema; 1, minimal erythema; 2, moderate erythema with sharply defined borders; 3, intense erythema with or without edema; 4, intense erythema with edema and blistering.

Sexual functioning and mood assessments were based on a questionnaire, one that had been validated for assessment of sexual function and mood and used previously in the evaluation of the effects of T gel on sexual function and mood (2). The questionnaire elicited information on sexual functions: performance, motivation, spontaneous erections, desire, enjoyment (with and without a partner), and satisfaction with erection duration and size. The sexual performance assessment was based on the following activities: orgasm, ejaculation, intercourse, masturbation, and erection in response to a sexual activity. The sexual performance score was the average number of days per 7-d week of these five activities. The sexual motivation assessment was based on the following activities: sexual daydreams, anticipation of sex, sexual interaction with partner, flirting by subject, and flirting by others toward subject. The sexual motivation score was the average number of days per 7-d week that these five activities occurred. The evaluation of spontaneous erections was the average number of days in a 7-d week that either spontaneous nighttime or daytime erections occurred. Sexual desire, sexual enjoyment, and satisfaction with erection were assessed on a Likert-type scale (score 0 to 7) and were calculated as average scores. Percentage of full erection was scored from 0% to 100%. Patients also rated positive mood (alert, full of energy, friendly, well or good) and negative mood (angry, irritable, sad or blue, tired, nervous) on a 0 to 7 categorical scale (0 = not at all true to 7 = very true). Average daily scores were computed.

Serum T and DHT levels were all measured at ICON Laboratories (Farmingdale, NY), using validated RIA kits. Kits (Diagnostic Products, Los Angeles, CA) were used for the T assays and kits obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX) were used for DHT assays. The lower limits of detection for the T and DHT pharmacokinetic assays were 0.1 nmol/liter (4 ng/ml) and 0.01 nmol/liter (4 pg/ml), respectively. The DHT assay had a 0.02% or less cross-reactivity (after solvent extraction) with T and T had a 3.3% or less cross-reactivity with DHT up to 173.6 nmol/liter (5000 ng/dl). The mean accuracy (recovery) of T determined by spiking steroid-free serum with varying amounts of T [1.5–1.3 nmol/liter (44–36 ng/dl)] was 98% (range, 93–103). The intra- and interassay coefficients of the T assay were 6.7% and 7.9% for a control group adult male range of 8.5–63.8 nmol/liter (245–1836 ng/dl). The mean accuracy (recovery) of DHT determined by spiking steroid-free serum with various amounts of DHT [0.2–1.4 nmol/liter (59–418 pg/ml)] was 94% (range, 85–130). The intra- and interassay coefficients of the DHT assay were 4.6% and 6.4%, respectively, for a control group adult male range of 0.3–2.4 nmol/liter (97–711 pg/ml).

Statistical analyses

The 24-h PK profiles for T and DHT were summarized by C_{avg} (AUC_{0-24} divided by the 24-h sampling period, where AUC is cal-

culated using the trapezoidal rule), the minimum postdose concentration (C_{min}), and the postdose C_{max} . The changes from baseline to d 30 and d 90 in C_{min} , C_{avg} , and C_{max} were analyzed using an analysis of covariance with baseline value as the covariate and treatment group as the factor. Patients randomized to AA2500 (50 mg/d or 100 mg/d) may have had their dose changed at the d 60 visit. Those patients with a dose change will be analyzed at d 90 using the dose they received at the d 60 visit. Similar analyses were used for the change from baseline in sexual function, mood, and body composition as well as for the clinical laboratory parameters at d 30, 60, and 90. Treatment-emergent adverse events were compared using a Fisher's exact test. Skin irritation at d 30, 60, and 90 was analyzed using a Wilcoxon rank sum test. At d 30 and 90, the 50 mg/d and 100 mg/d AA2500 treatment groups were compared with T patch (PK parameters, sexual function, body composition, and mood) and placebo (sexual function, body composition, and mood). For each comparison, including safety parameters, an α -value of 0.05 was considered significant. The changes from baseline in sexual function, body composition, and mood were also analyzed for nonzero differences within each treatment group based on the adjusted least squares means from the analysis of covariance model. SAS version 6.12 (SAS Institute, Inc., Cary, NC) was used for all analyses. All data in tables are presented as means (\pm sd).

Results

Subjects

A total of 406 patients were randomized with 99, 106, 102, and 99 being randomized to the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively (Table 1). Baseline patient characteristics (age, height, weight, body mass index, serum T at screening, I-PSS scores, and PSA levels) were comparable. Of the patients who had a valid PK profile, 70 of 399 (17.5%, 21 patients in 50 mg/d AA2500, 20 patients in 100 mg/d AA2500, 16 patients in T patch, and 13 patients in placebo groups) had a C_{avg} above 10.4 nmol/liter at baseline. Of these patients, 71% had at least one or more serum T measurement less than 10.4 nmol/liter during the course of d -1. Baseline mean C_{avg} serum T concentrations were 12.6, 12.1, 12.8, and 12.4 nmol/liter in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively. Fifty percent of the patients were aged 58 yr or older and approximately 26% were aged 65 yr and older with a mean age of 58 yr. Patient hypogonadism was primarily attributed to the secondary cause of aging and normogonadotrophic hypogonadism; these cases accounted for 91% of all causes in the overall population (Table 1). A significant proportion of enrolled patients completed the 90-d study (90% and 92% in the AA2500 and placebo groups, respectively, and 75% in the T patch group). The primary reason for the higher rate of discontinuations in the T patch group was adverse events (17%) with the majority of events being related to skin irritations at the patch site. Titrations made at d 60 in the AA2500 T gel groups were: 52 patients started on 50 mg/d dose and remained on 50 mg/d dose for the entire study, 43 patients who started on 50 mg/d dose were titrated up to the 100 mg/d dose, 93 patients who started on 100 mg/d dose remained at the 100 mg/d dose for the entire study, and 4 patients who started on 100 mg/d dose were titrated down to 50 mg/d dose. Patients who remained at 50 mg were more likely to have secondary hypogonadism, excluding aging, than patients who titrated to 100 mg. Dosing compliance ranged from 94.9% (placebo) to

97.1% (50 mg/d AA2500 and 100 mg/d AA2500 combined analysis).

T pharmacokinetics (Fig. 1 and Table 2)

At baseline, mean C_{avg} serum T concentrations were below the normal adult range (10.4–34.7 nmol/liter) and similar across treatment groups. By d 30, the mean C_{avg} for the 50 mg/d AA2500 treatment had increased 50% over baseline with a similar increase being evidenced in the T patch treatment group. The 100 mg/d AA2500 dose resulted in a 173% increase with a significant difference ($P < 0.001$) in comparison with the T patch treatment group. The C_{avg} was increased above 10.4 nmol/liter in 55% of 50 mg/d AA2500 patients, 95% of the 100 mg/d AA2500 patients, 68% of the T patch patients, and 8% of the placebo patients. In the 100

mg/d AA2500 group, 30 patients had a C_{max} higher than the upper limit of normal (34.7 nmol/liter), but 26 of these patients had a C_{avg} still between 10.4–34.7 nmol/liter. The degree of fluctuation during a day in serum values [$(C_{max} - C_{min})/C_{avg}$] was significantly smaller in the two AA2500 dose groups in comparison with the T patch group. By d 90, similar results were seen across the treatment groups. Approximately 75% of 50 mg/d AA2500 and 80% of 100 mg/d AA2500 treated patients had C_{avg} values above 10.4 nmol/liter, in comparison with 57% of the T patch-treated patients and 10% of placebo-treated patients.

DHT pharmacokinetics (Fig. 2 and Table 3)

At baseline, mean C_{avg} serum DHT concentrations were below the normal adult male range (0.9–2.6 nmol/liter) and similar across treatment groups. Mean changes in DHT C_{avg} from baseline to d 30 for the 50 mg/d AA2500 and 100 mg/d AA2500 dose groups were more than 4- and 7-fold greater, respectively, than changes observed in the T patch treatment group ($P < 0.001$ for each comparison). Similar to C_{avg} , C_{min} results further demonstrated the effectiveness of both AA2500 doses in increasing the d 30 C_{min} to a significantly greater degree than the T patch treatment ($P < 0.001$ for each comparison). The 100 mg/d AA2500 dose achieved a mean d 30 C_{min} that was within the normal range. For C_{max} , the d 30 effects reported were similar to that observed with C_{avg} . The C_{max} mean changes in serum DHT from baseline to d 30 for both AA2500 dose groups were approximately 4- and 7-fold greater than that evidenced in the T patch group ($P < 0.001$ for each comparison) with 15 (16%), 39 (42%), 1 (1%), and no patients in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively, exceeding the normal range. At d 30, examination of the DHT to T C_{avg} ratio demonstrated that this ratio was not altered by either the T patch or placebo treatment, whereas a near doubling of the ratio was, respectively, evidenced with both AA2500 doses. By d 90, similar results were seen across the treatment groups. Additionally, the higher serum levels of DHT obtained with the AA2500 treatments correlated with the serum T levels obtained.

Body composition (Fig. 3)

At baseline, there were no significant differences in LBM, FM, %F, and TBM among the four treatment groups. At d 90, the 100 mg/d AA2500 treatment increased LBM to a significantly greater degree than the T patch or placebo treatment ($P < 0.05$ for each comparison) with mean changes from baseline of 1.5 ± 4.5 , 1.7 ± 2.6 , 0.9 ± 1.8 , and 0.6 ± 1.8 kg for the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively. With the exception of placebo treatment, all treatments resulted in a decrease in FM, which were significant, compared with placebo ($P < 0.01$). Reductions of 0.8 ± 2.4 , 0.8 ± 2.0 , 0.4 ± 1.8 , and 0.1 ± 1.5 kg were noted in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively. Reductions in %F were evidenced in all treatment groups, with the AA2500 treatments yielding the most notable decreases. Specifically, at d 90, the 50 mg/d AA2500 dose resulted in a reduction of $1.1 \pm 3.2\%$ which was sig-

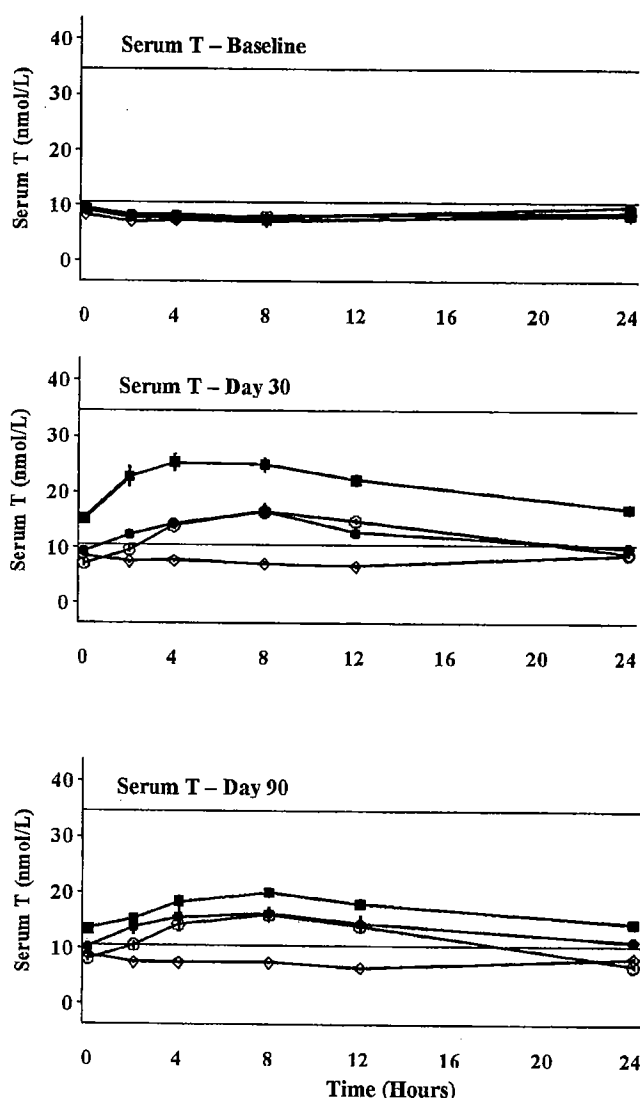


FIG. 1. Serum T concentrations before (baseline) and after study drug treatment on d 30 and 90. Time 0 was approximately 0800 h. Solid horizontal lines denote the adult male range (10.4–34.7 nmol/liter). ●, 50 mg/d AA2500; ■, 100 mg/d AA2500; ○, T patch; ◇, placebo.

TABLE 2. T (nmol/liter): mean d 30 and 90

| | | AA2500 | | T patch | Placebo |
|--------|------------------|----------|--------------------------|--------------------------|------------|
| | | 50 mg/d | 100 mg/d | | |
| Day 30 | | | | | |
| | C _{avg} | Baseline | 8.6 ± 2.8 | 7.8 ± 2.8 | 8.2 ± 2.8 |
| | | Actual | 12.7 ± 6.5 | 21.3 ± 9.9 ^b | 12.7 ± 4.2 |
| | C _{min} | Baseline | 6.8 ± 2.4 | 6.2 ± 2.6 | 6.7 ± 2.3 |
| | | Actual | 7.7 ± 4.4 ^a | 13.6 ± 6.5 ^b | 6.2 ± 2.9 |
| | C _{max} | Baseline | 10.7 ± 3.6 | 9.9 ± 3.2 | 10.2 ± 3.7 |
| Day 90 | | Actual | 18.8 ± 12.9 ^a | 31.2 ± 19.8 ^b | 18.8 ± 6.9 |
| | C _{avg} | Baseline | 9.2 ± 3.4 | 7.7 ± 2.4 | 8.3 ± 2.8 |
| | | Actual | 13.8 ± 8.1 | 17.1 ± 8.2 ^b | 11.9 ± 4.6 |
| | C _{min} | Baseline | 7.4 ± 2.8 | 6.1 ± 2.3 | 6.7 ± 2.1 |
| | | Actual | 8.7 ± 3.9 ^b | 10.9 ± 6.0 ^b | 5.7 ± 2.8 |
| | C _{max} | Baseline | 11.3 ± 4.1 | 9.8 ± 2.9 | 10.3 ± 3.7 |
| | | Actual | 19.5 ± 12.2 | 24.4 ± 13.8 ^b | 18.5 ± 8.2 |

Values are expressed as means ± 1 SD. Change from baseline significant vs. T patch: ^a $P < 0.05$, ^b $P < 0.001$.

nificant in comparison to the reduction evidenced with placebo treatment ($0.2 \pm 1.4\%$, $P < 0.05$). Furthermore, the 100 mg/d AA2500 treatment resulted in a $1.2 \pm 1.9\%$ reduction at d 90, which was not only significant in comparison with placebo ($P < 0.01$) but also significant in comparison with the T patch treatment ($0.5 \pm 1.6\%$, $P < 0.05$). Although all treatments resulted in minimal increases in TBM, no significant differences were noted among the treatment groups.

Mood and sexual function (Table 4)

Although all treatments resulted in mean improvements from baseline in both positive and negative mood scores, no significant differences among the treatment groups were observed.

At baseline, sexual function scores were similar across the four treatment groups. Evaluation of the mean data demonstrated that the 100 mg/d AA2500 dose showed a significant improvement at d 90 over placebo treatment for spontaneous erections ($P < 0.001$), sexual motivation ($P < 0.05$), sexual desire ($P < 0.01$), and sexual performance ($P < 0.05$). Furthermore, the improvement from baseline was also significant for these parameters.

All other measures of sexual function (e.g. sexual enjoyment with a partner, sexual enjoyment without a partner, satisfaction with erection duration, and percentage of full erection) showed no significant difference in improvement between treatment groups.

Safety

Adverse events. The incidence of treatment-related adverse events was 29.1%, 36.9%, 62.7%, and 40.4% in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively. Although treatment in the AA2500 and placebo groups was relatively well tolerated over the 90-d study period, the T patch-treated patients experienced a substantially higher rate of adverse events. Those most commonly seen were application site erythema, application site rash, application site pruritus, application site reactions, and application site irritation.

Specific events that were possibly or probably related to study drug and reported by 1% or more of the AA2500

patients and greater than placebo included application site reactions, BPH, increases in blood pressure and hematocrit/hemoglobin, gynecomastia, headache, hot flushes, insomnia, increased lacrimation, mood swings, smell and taste disorders, and spontaneous penile erections.

Only six patients in AA2500 treatment groups experienced adverse events that led to discontinuation. Specific events in the 100 mg/d AA2500 treatment group included vertigo, coronary artery disease, depression with suicidal ideation, urinary tract infection/pneumonia, and hypertension. All events with the exception of hypertension were considered unrelated to treatment. Mood swings, considered related to treatment, was the only event in the 50 mg/d AA2500 treatment group that led to discontinuation. Lastly, no patients in the 50 mg/d or 100 mg/d AA2500 treatment groups discontinued because of skin reaction, whereas the majority of patients that discontinued in the T patch group did so as a result of local dermal site reactions ($n = 15$).

With regard to prostate-related events, mild BPH was reported in two patients in the 100 mg/d AA2500 treatment group and one patient in the placebo treatment group. Additionally, two T patch-treated patients were diagnosed with prostatic cancer.

Laboratory analyses

Statistically significant differences between the 50 mg/d and/or 100 mg/d AA2500 groups and placebo groups in serum blood urea nitrogen (-2.0 ± 4.0 , -1.7 ± 4.3 , and -0.6 ± 3.9 mg/dl, respectively), creatinine (0.04 ± 0.12 , 0.07 ± 0.14 , and -0.02 ± 0.12 mg/dl, respectively), and fasting glucose levels (-2.2 ± 18.1 , -5.6 ± 25.1 , and 4.0 ± 25.3 mg/dl, respectively) were observed; however, these differences were minor and not clinically meaningful.

At d 90, clinically notable decreases from baseline in average total cholesterol (TC), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were evidenced with the 100 mg/d AA2500 group (-7% , -7% , and -8% , respectively). Mean d 90 LDL/HDL ratios (2.73, 2.56, 2.52, and 2.41 for the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively) remained essentially unchanged from baseline. Additionally, mean d 90 HDL/TC

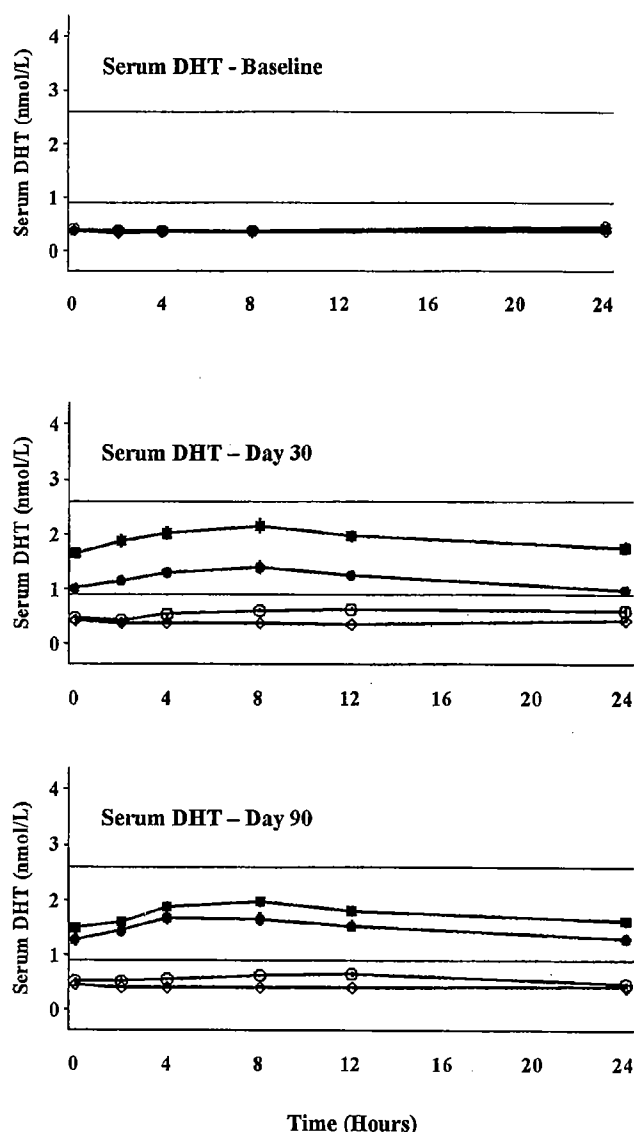


FIG. 2. Serum DHT concentrations before (baseline) and after study drug treatment on d 30 and 90. Time 0 was approximately 0800 h. Solid horizontal lines denote the adult male range (0.8–2.6 nmol/liter). ●, 50 mg/d AA2500; ■, 100 mg/d AA2500; ○, T patch; ◇, placebo.

ratios (0.23, 0.24, 0.24, and 0.24 for the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively) also remained unchanged.

Increases in hemoglobin and hematocrit are known pharmacological class effects of T. Consistent with this, patients in the 50 mg/d and 100 mg/d AA2500 group experienced statistically significant mean d 90 increases in hematocrit and hemoglobin of $2.3 \pm 3.4\%$ and 0.96 ± 0.96 g/dl, respectively, in the 50 mg/d AA2500 group and $2.8 \pm 3.5\%$ and 0.94 ± 1.06 g/dl in the 100 mg/d AA2500 group, compared with the placebo treatment group ($-0.1 \pm 2.8\%$ and 0.12 ± 0.71 g/dl) and T patch treatment group ($1.1 \pm 2.6\%$ and 0.48 ± 0.74 g/dl). The effects observed with T patch treatment were consistently greater than observed with placebo treatment

but less than those observed with the AA2500 treatments, reflecting the lower average serum T levels associated with the T patch treatment. At d 30 and 60, similar effects were reported for the AA2500 treatment group. Overall, approximately 3%, 6%, 1%, and 1% of patients in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively, experienced a hematocrit value more than 55% at least once during the study. Statistically significant mean changes were also reported for lymphocytes and monocytes, but the changes were small and not of apparent clinical significance.

PSA values (Table 5) more than 4.0 ng/ml were noted at least once during treatment in 1.8%, 2.9%, 6.6%, and 3.2% of patients in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively. PSA elevations were noted in all groups with the T patch group evidencing the greatest number of transient and persistent elevations. Mean changes from baseline to d 90 of 0.3 ± 1.8 , 0.1 ± 0.4 , 0.2 ± 0.6 , and -0.1 ± 0.4 ng/ml for the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively. Of note, one patient in the 50 mg/d AA2500 treatment group experienced a transient elevation of 17.6 ng/ml (without clinically valid explanation) that upon repeat evaluation returned to normal (3.6 ng/ml).

The changes from baseline for I-PSS were small, and the incidence of patients experiencing a general worsening of their DRE was low (3.4%, 1.4%, 0%, and 4.2% in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively). Both parameters provided no evidence of clinically relevant treatment-related effects or differences.

Skin irritation (Fig. 4)

Figure 4 provides a graphic illustration of the frequency distribution of patients having positive skin irritation scores. It can be seen that the events occurred predominantly in the T patch treatment group and only a few mild reactions occurred in the combined AA2500 and placebo treatment groups. Additionally, the figure illustrates that the T patch acted as an irritant in some patients who experienced classic signs of contact dermatitis and that the AA2500 treatments were no more irritating than the placebo gel vehicle from d 60 through the completion of the study.

Discussion

This study demonstrated that this new, unique T gel (AA2500), when titrated to clinical effectiveness, was superior to the T patch in normalizing serum T in patients with hypogonadism. Specifically, the 100 mg/d dose was not only effective in significantly improving sexual performance, sexual motivation, and sexual desire and increasing spontaneous erections but also increasing LBM and decreasing FM and %F. There were associated increases in hemoglobin and hematocrit, which are known pharmacological class effects of T administration. The small mean increases in PSA observed with AA2500 doses and the T patch treatment groups, but not the placebo group, were not associated with an increase in I-PSS. Small decreases in TC, LDL, and HDL were observed in the AA2500 treatment groups with no changes in HDL/TC or LDL/HDL ratios. Erythema, rash, pruritus,

TABLE 3. DHT (nmol/liter): mean d 30 and 90

| | | AA2500 | | T patch | Placebo |
|-----------|-----------|-------------------|-------------------|-----------------|-----------------|
| | | 50 mg/d | 100 mg/d | | |
| Day 30 | C_{avg} | Baseline | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.2 |
| | Actual | 1.2 ± 0.7^a | 1.9 ± 1.0^a | 0.6 ± 0.3 | 0.4 ± 0.2 |
| C_{min} | Baseline | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 |
| | Actual | 0.8 ± 0.6^a | 1.4 ± 0.9^a | 0.4 ± 0.2 | 0.3 ± 0.2 |
| C_{max} | Baseline | 0.5 ± 0.2 | 0.5 ± 0.3 | 0.5 ± 0.3 | 0.5 ± 0.2 |
| | Actual | 1.7 ± 1.0^a | 2.6 ± 1.4^a | 0.8 ± 0.7 | 0.5 ± 0.3 |
| DHT/T† | Baseline | 0.05 ± 0.02 | 0.05 ± 0.03 | 0.05 ± 0.02 | 0.06 ± 0.04 |
| | Actual | 0.09 ± 0.04^a | 0.09 ± 0.04^a | 0.05 ± 0.02 | 0.06 ± 0.04 |
| Day 90 | C_{avg} | Baseline | 0.5 ± 0.2 | 0.4 ± 0.2 | 0.4 ± 0.2 |
| | Actual | 1.5 ± 0.7^a | 1.8 ± 0.9^a | 0.6 ± 0.3 | 0.4 ± 0.2 |
| C_{min} | Baseline | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| | Actual | 1.0 ± 0.6^a | 1.2 ± 0.7^a | 0.3 ± 0.2 | 0.3 ± 0.2 |
| C_{max} | Baseline | 0.6 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.2 |
| | Actual | 2.0 ± 0.9^a | 2.3 ± 1.2^a | 0.8 ± 0.4 | 0.5 ± 0.3 |
| DHT/T† | Baseline | 0.05 ± 0.03 | 0.05 ± 0.02 | 0.05 ± 0.02 | 0.06 ± 0.04 |
| | Actual | 0.11 ± 0.04^a | 0.10 ± 0.04^a | 0.05 ± 0.03 | 0.06 ± 0.05 |

Values are expressed as means \pm 1 SD. †DHT/T = ratio of C_{avg} of DHT and T (nmol/liter units do not apply).

^a $P < 0.001$. Change from baseline significant vs. T patch.

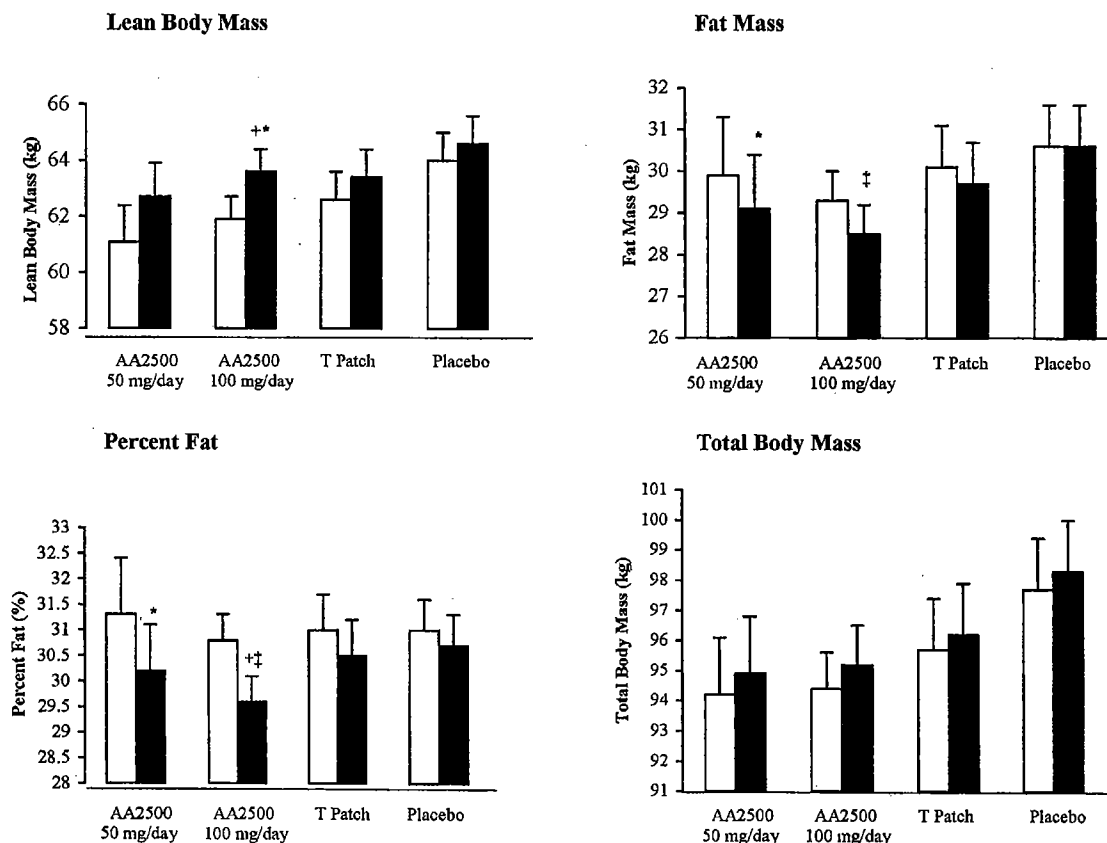


FIG. 3. Values are expressed as means \pm 1 SE in LBM, FM, %F, and TBM after treatment with 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo. □, Baseline; ■, d 90; *, Significant vs. placebo: $P < 0.05$; +, significant vs. T patch: $P < 0.05$; †, significant vs. placebo: $P < 0.01$.

application site reactions, and irritation were observed much more frequently in the T patch group, compared with both AA2500 treatment groups and the placebo treatment group. Additionally, the T patches acted as an irritant in some patients who experienced classic signs of contact dermatitis,

whereas the AA2500 gel treatments resulted in minimal skin erythema in only a few patients with the incidence being similar to that observed in the placebo treatment group.

The 100 mg/d AA2500 treatment increased LBM to a greater degree than either the T patch or placebo, and both

TABLE 4. Sexual function scores: mean change from baseline to d 90

| | | AA2500 | | T patch | Placebo |
|---|----------|------------------------|--------------------------|------------------------|------------------------|
| | | 50 mg/d | 100 mg/d | | |
| Spontaneous erections (average weekly) | Baseline | 0.7 ± 0.9 | 0.8 ± 1.1 | 1.0 ± 1.3 | 1.0 ± 1.2 |
| | Change | 0.3 ± 1.3 | 0.7 ± 1.4 ^{e,f} | 0.3 ± 1.1 | 0.0 ± 1.0 |
| Motivation (average weekly) | Baseline | 1.6 ± 1.5 | 1.8 ± 1.4 | 1.6 ± 1.2 | 1.5 ± 1.2 |
| | Change | 0.2 ± 1.5 | 0.6 ± 1.4 ^{a,f} | 0.4 ± 1.1 ^d | 0.1 ± 1.2 |
| Desire (average daily) | Baseline | 2.3 ± 1.4 | 2.4 ± 1.4 | 2.2 ± 1.4 | 2.1 ± 1.4 |
| | Change | 0.5 ± 1.2 ^e | 1.0 ± 1.4 ^{b,f} | 0.6 ± 1.2 ^f | 0.5 ± 1.0 ^f |
| Performance (average weekly) | Baseline | 0.8 ± 0.9 | 0.8 ± 0.9 | 0.7 ± 0.8 | 0.8 ± 0.8 |
| | Change | 0.3 ± 1.1 | 0.5 ± 1.2 ^{a,f} | 0.3 ± 0.7 ^d | 0.2 ± 0.9 ^d |

Values are expressed as means ± 1 SD. Significant *vs.* placebo: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$. Significant within treatment group change from baseline: ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$.

TABLE 5. PSA: summary of changes to d 90 and classification of overall elevations

| | AA2500 | | T patch | Placebo |
|-----------------------------|------------------------|-----------|-----------|------------|
| | 50 mg/d | 100 mg/d | | |
| Baseline (ng/ml) | 1.2 ± 1.0 | 1.2 ± 0.9 | 1.4 ± 1.1 | 1.1 ± 1.0 |
| Change (ng/ml) | 0.3 ± 1.8 ^a | 0.1 ± 0.4 | 0.2 ± 0.6 | −0.1 ± 0.4 |
| PSA elevations ^b | | | | |
| >4.0 ng/dl | 1 | 4 | 6 | 3 |
| Transient | 1 | 2 | 2 | 1 |
| Persistent | 0 | 2 | 4 | 2 |

Baseline and change values are expressed as means ± 1 SD.

^a $P < 0.01$, significant *vs.* placebo.

^b Number of subjects experiencing at least one PSA value >4.

Skin Irritation Scores

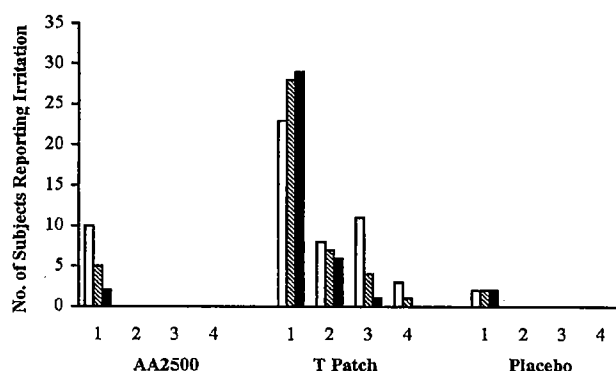


FIG. 4. Distribution of subjects with positive application site/skin irritation scores on d 30, 60, and 90. 1, Minimal erythema; 2, moderate erythema with sharply defined borders; 3, intense erythema with or without edema; 4, intense erythema with edema and blistering/erosion. The 50 mg/d and 100 mg/d AA2500 treatment groups combined. □, d 30; ▨, d 60; ■, d 90.

doses of AA2500 also resulted in a significantly greater decrease in FM and %F, compared with placebo. Normalization of serum T levels were achieved with both doses of AA2500 for average T levels and minimum T levels over a 24-h dosing period. In contrast, the T patch group was able to restore only average T levels. Previously reported data suggest that increases in LBM and decreases in FM are correlated with serum T levels (1). This difference in normalization may explain the greater increase in LBM and greater decrease in FM and %F observed with the AA2500 treatments. In previous studies in hypogonadal men, T replacement therapy has resulted in decreases in FM in some studies using injectable or transdermal T but not in other studies in which

either injectable or sublingual T has been administered. The difference in results observed in these previous reports might be due to lower serum T levels achieved by different T preparations.

Regarding DHT, although it is true that the AA2500 treatments produced higher serum levels at d 30 and 90, the DHT/T ratio remained stable and similar to that reported in normal men, demonstrating concordance with the naturally occurring 5 α -reductase conversion of T to DHT. The effect of serum DHT levels on the intraprostatic levels of DHT is not known. As with serum T levels, a prospective relationship between DHT serum levels and the incidence of prostate cancer has not been demonstrated (10, 11). Further long-term studies are needed to clarify the effect of increased DHT on the prostate.

There were no unexpectedly abnormal laboratory values and the incidence of clinically relevant abnormal findings was low. By d 90, patients in the AA2500 group who were administered 50 mg/d or 100 mg/d experienced statistically significant increases in hematocrit and hemoglobin, compared with patients receiving T patch or placebo. The increases observed in the T patch group were consistently greater than those observed in the placebo group but less than those observed in the AA2500 groups. This is likely a result of the lower serum T levels achieved with T patch. A previous study demonstrated similar increases in hematocrit and hemoglobin with T replacement therapy, with these increases being more marked with higher doses of T (1). These small increases in hematocrit and hemoglobin, which can occur with T replacement therapy, may even be beneficial in hypogonadal patients in whom anemia, lethargy, and fatigue are commonly found; however, a small percentage of treated individuals may increase their hematocrit levels to more than 55% and in turn be prone to the problems

associated with polycythemia. To this end, periodic monitoring of hematocrit is recommended to determine whether T therapy dose adjustments or termination (*i.e.* in the event hematocrit values do not fall below 55%) may be required.

Small increases in PSA similar to those seen in previous studies with T replacement therapy were observed in both AA2500 groups and in the T patch group. However, the magnitude of the increase in PSA, changes in I-PSS, and findings from DRE following treatment with either dose of AA2500 or T patch in this study were not of clinical concern. Although T has not been shown to induce cancer of the prostate, two patients on the T patch were diagnosed with prostate cancer during the study. This is not surprising because elderly men are at an increased risk of developing prostate cancer and the diagnosis can be made as a result of an elevated PSA subsequently leading to prostatic biopsy.

Very few adverse effects were reported following topical application of AA2500, and those that were reported were similar in type to the known class effects of T. Of particular note was the very low incidence of skin irritation reported with AA2500, which was comparable to placebo and significantly lower than T patch. Furthermore, no patient in the gel groups discontinued because of skin intolerance.

This study clearly shows that 100 mg/d AA2500 dose is superior to T patch in normalizing serum T and DHT in hypogonadal men. The AA2500 treatments resulted in increasing LBM (100 mg/d dose) and decreasing FM and %F to a greater degree than either the T patch or placebo. Furthermore, significant improvements from baseline and in comparison to placebo were observed for spontaneous erections, sexual motivation, sexual desire, and sexual performance with 100 mg/d AA2500 dose. Overall, this new, unique T gel (AA2500) can offer benefit over other transdermal preparations because of improved 24-h serum T levels and improved compliance as a result of a lower incidence of local dermal irritation.

Acknowledgments

We thank Drs. John Amory and William Bremner (University of Washington, Seattle, WA) for reviewing the manuscript and providing editorial guidance; Gary Nevins, D.C., for management of the clinical study; and Allen Secrest, B.Sc., for preparation of the manuscript.

Received July 8, 2002. Accepted March 5, 2003.

Address all correspondence and requests for reprints to: Christopher Steidle, M.D., Northeast Indiana Research, LLC, 2512 East DuPont Road, Suite 100, Fort Wayne, Indiana 46825. E-mail: chrisste@aol.com.

This work was supported by Auxilium Pharmaceuticals, Inc.

The North American AA2500 T Gel Study Group includes: M. Bas-tuba (San Diego Urology, La Mesa, CA); W. Borkon (Park Nicollet Clinic, St. Louis Park, MN); M. Borofsky (Clinical Research Center of Reading, West Reading, PA); P. Butler (Coast Urology Medical Group, Inc., La Jolla, CA); J. Caldwell (Radiant Research, Inc., Gainesville, FL); C. Cascione (Gainesville VAMC, Gainesville, FL); C. Corder (COR Clinical Research, Oklahoma City, OK); Z. Dalu (Radiant Research, Inc., St. Louis, MO); T. Decker (Strafford Medical Association, Dover, NH); L. Galitz (South Florida Bioavailability Clinic, Miami, FL); E. Gillie (ICSL

Clinical Studies, Fort Myers, FL); G. Gollapudi (Diabetes Center of the Southwest, Midland, TX); P. Hatcher (Volunteer Research Group, Knoxville, TN); K. Jacoby (Integrity Medical Research, Seattle, WA); M. Jayson (Medical & Clinical Research Associates, Bayshore, NY); W. Jones (Radiant Research, Inc., Boise, ID); L. Karsh (Western Urologic Research Center, Wheat Ridge, CO); J. Kaufman (Urology Research Options, Aurora, CO); W. Keating (SFM Clinical Trials, Scotland, PA); E. Killorin (Sandy Springs Urology, Atlanta, GA); T. Marbury (Orlando Clinical Research Center, Orlando, FL); J. McMurray (Medical Affiliated Research Center, Inc., Huntsville, AL); K. Meissner (Urology Consultants, San Antonio, TX); Y. Moy (The Connecticut Clinical Research Center/Urology Specialists, Waterbury, CT); T. Mulligan (McGuire VAMC Richmond, Richmond, VA); J. Newman (Quality Care Medical Center, Vista, CA); H. Offenber (Radiant Research, Inc., Daytona Beach, FL); M. Parker (Radiant Research, Inc., Tucson, AZ); R. Pearson (Urology Center, Memphis, TN); T. Phillips (Metrolina Medical Research, Charlotte, NC); W. Pittman (Urology Centers of Alabama, Homewood, AL); R. Rollins (Southeastern Urological Center, Tallahassee, FL); G. Salazar (Sun Research Institute, San Antonio, TX); S. Schwartz (Diabetes and Glandular Disease Research Associates, San Antonio, TX); R. Sievers (Wells Institute for Health Awareness, Kettering, OH); F. Snoy (Urology Group of New Mexico, Albuquerque, NM); J. Soufer (Phoenix Internal Medicine Associates, Waterbury, CT); C. Steidle (Northeast Indiana Research, Fort Wayne, IN); E. Stulberger (Physicians in Urology, Livingston, NJ); J. Susset (MultiMed Research, Providence, RI); M. Vance (University of Virginia Health System, Charlottesville, VA); J. Walton (Radiant Research, Inc., Greer, SC); W. Wells, Jr. (Alabama Research Center, Birmingham, AL).

References

1. Wang C, Swerdloff RS, Iranmanesh A, Dobs A, Snyder PJ, Cunningham G, Matsumoto M, Weber T, Berman N, Testosterone Gel Study Group 2000 Transdermal testosterone gel improves sexual function, mood, muscle strength, and body composition parameters in hypogonadal men. *J Clin Endocrinol Metab* 85:2839–2853
2. Arver S, Dobs AS, Meikle AW, Caramelli KE, Rajaram L, Sanders SW, Mazer NA 1997 Long-term efficacy, and safety of a permeation-enhanced testosterone transdermal system in hypogonadal men. *Clin Endocrinol (Oxf)* 47:727–737
3. Behre HM, von Eckardstein S, Kliersch S, Nieschlag E 1999 Long-term substitution therapy of hypogonadal men with transscrotal testosterone over 7–10 years. *Clin Endocrinol (Oxf)* 50:629–635
4. Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Holmes JH, Dlewati A, Staley J, Santanna J, Kapoor SC, Attie MF, Haddad Jr JG, Strom BL 1999 Effect of testosterone treatment on bone mineral density in men over 65 years of age. *J Clin Endocrinol Metab* 84:1966–1972
5. Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Lenrow DA, Holmes JH, Dlewati A, Santanna J, Rosen CJ, Strom BL 1999 Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. *J Clin Endocrinol Metab* 84:2647–2653
6. Jordan Jr WP, Atkinson LE, Lai C 1998 Comparison of the skin irritation potential of two testosterone transdermal systems: an investigational system and a marketed product. *Clin Ther* 20:80–87
7. Jordan Jr WP 1997 Allergy and topical irritation associated with transdermal testosterone administration: a comparison of scrotal and nonscrotal transdermal systems. *Am J Contact Dermatitis* 8:108–113
8. Marbury T, Hamill E, Bachand R, Seebree T, Smith T 2003 Evaluation of the pharmacokinetic profiles of the new testosterone topical gel formulation, Testim compared to AndroGel. *Biopharm Drug Dispos* 24:115–120
9. Dobs AS, Meikle AW, Arver S, Sanders SW, Caramelli KE, Mazer NA 1999 Pharmacokinetics, efficacy, and safety of a permeation-enhanced testosterone transdermal system in comparison with biweekly injections of testosterone enanthate for the treatment of hypogonadal men. *J Clin Endocrinol Metab* 84:3469–3478
10. Gann PH, Hennekens CH, Ma J, Longcope C, Stampfer MJ 1996 Prospective study of sex hormone levels and risk of prostate cancer. *J Natl Cancer Inst* 88:1118–1126
11. Mohr BA, Feldman HA, Kalish LA, Longcope C, McKinlay JB 2001 Are serum hormones associated with the risk of prostate cancer? Prospective results from the Massachusetts Male Aging Study. *Urology* 57:930–935

Detection of anabolic steroid administration: ratio of urinary testosterone to epitestosterone vs the ratio of urinary testosterone to luteinizing hormone

PAUL J. PERRY,^{1,3*} JOHN H. MACINDOE,² WILLIAM R. YATES,¹ SHANE D. SCOTT,³ and
TIMOTHY L. HOLMAN¹

Our goal in this study was to determine whether the urinary ratio of testosterone to luteinizing hormone (T/LH) as an indicator of exogenous anabolic steroid (AS) use is superior to the urinary ratio of testosterone to epitestosterone (T/E). After 2 weekly placebo injections, 19 subjects were given testosterone cypionate (TC) injections of 250 or 500 mg/week for 14 weeks followed by 14 weekly placebo injections. Patients were considered to have ceased taking TC if they tested negative 9 weeks after their last injection. For detection of illicit or supraphysiological TC (AS) use, the urinary T/E ratio of ≥ 6 yielded a false-negative rate of 46% and a false-positive rate of 4%. However, a urinary T/LH ratio of ≥ 30 produced a false-negative rate of only 24% and a false-positive rate of 13%. We conclude that the urinary T/LH ratio of ≥ 30 is a more sensitive marker of AS use than the urinary T/E ratio of ≥ 6 and remains sensitive for twice as long as urinary T/E.

INDEXING TERMS: abused drugs • sports medicine • GC-MS • androgens • anabolic steroids

The primary method for detecting illicit anabolic steroid (AS) use has been the analysis of urinary steroids.⁴ This methodology has been successful for the majority of steroids, especially the synthetic variety that have specific

structures that are easily identified by GC-MS. However, the detection and monitoring of anabolic compounds is not fail-safe. Detection of the illicit use of testosterone (T), a naturally occurring AS, has become a difficult clinical problem. Methods for detecting administration of exogenous T depend on distortions of the normal hormone profile in the user's urine [1]. Attempts to identify optimal markers of exogenous T administration from untimed urine samples in male athletes have uncovered several compounds as possible indicators of T abuse. In 1982, the ratio of androgen glucuronides to epitestosterone (E; 17 α -hydroxy-4-androsten-3-one) was adopted by the Medical Commission of the International Olympic Committee (IOC) in Los Angeles, with a cutoff point ≥ 6 being the sole test for illicit T self-administration [2, 3]; the expected urinary ratio of T/E among healthy subjects not using AS is ~ 1 [1]. However, analyses from all IOC-accredited laboratories in 1991 suggested that the majority of athletes who were using AS had switched from synthetic compounds to T pharmaceuticals to evade detection [4]. Consequently, covert AS use has become more difficult to detect.

The overall incidence of urinary T/E ≥ 6 in the general population of healthy males not abusing steroids is $<0.8\%$, as evaluated by Catlin and Hatton [5] and confirmed by Dehennin [4]. In general, the increase of the T/E ratio after high-dose T administration results from increased T excretion and a decrease of E output [6]. However, some athletes have produced false-positives, i.e., T/E ratios ≥ 6 with subsequent verification that no exogenous T had been administered [7]. Dehennin and Matsumoto [6] indicated that this problem could be reduced by taking into account the sulfate excretions of E (ES) in the ratio T/(ES + E), the relevant threshold being 2.85. Accordingly, Dehennin [4] suggested that using a

Departments of ¹ Psychiatry and ² Internal Medicine, College of Medicine, and ³ Department of Clinical Pharmacy, College of Pharmacy, University of Iowa, Iowa City, IA 52246

*Address correspondence to this author, at: S415 Pharmacy, University of Iowa, Iowa City, IA 52246. Fax 319-353-5646; e-mail paul-perry@uiowa.edu.

⁴ Nonstandard abbreviations: AS, anabolic steroid(s); T, testosterone; E, epitestosterone; IOC, International Olympics Committee; ES, epitestosterone sulfate; LH, luteinizing hormone; and TC, testosterone cypionate.

Received September 10, 1996; revised December 3, 1996; accepted December 20, 1996.

T/(ES + E) ratio of ≥ 3.0 would be a more sensitive marker of covert T use.

Dehennin [7] also noted that the joint misuse of T and E could also lead to false-negative test results, and the IOC in 1992 recommended that urinary E concentrations >150 $\mu\text{g/L}$ should be noted as abnormally high and therefore suspicious. False-negative results can also be produced by stimulation of testicular steroidogenesis by administering human chorionic gonadotropin, which would result in a concomitant increase in the urinary excretion rate of T and E but with no significant change in the T/E ratio [1]. Dehennin and Matsumoto [6] confirmed earlier reports of false negatives by demonstrating that, despite their determination that an average dose of 47 mg of exogenous testosterone per week would equal or exceed the IOC cutoff, 2 of 9 subjects receiving 72 mg of testosterone (100 mg of testosterone enanthate) per week for 6 months did not produce a T/E ratio ≥ 6 .

Dehennin [4] suggested that when a T/E ratio of 6 to 12 is found for the first time in subjects for whom no documentation of a previously normal ratio exists, some complimentary criteria should be examined. He found that the ratio of urinary T and E glucuronides to 5-androstene-3 β ,17 α -diol glucuronide was increased in the use of exogenous T and E use despite the T/E ratio being <6 . These findings indicate a need for further study of additional markers for detecting the administration of T.

Because the secretion of T is under the control of luteinizing hormone (LH), Brooks et al. [8] suggested that the urinary T/LH ratio might be a potentially useful marker for detecting administration of exogenous T. Kicman et al. [9] observed that high-dose T administration resulted in dose-dependent suppression of both serum and urinary LH. This was confirmed by Matsumoto [2], who found that the urinary LH excretion was reduced to a lesser extent than was the decrease in both E and T conjugates, such that the T/LH values were lower than those reported by Kicman et al.—also suggesting a need for more study.

Palonek et al. [3] reported significantly increased T/LH ratios in 11 healthy sedentary men participating in a WHO investigational program for male contraception. Each subject received 144 mg of T per week (200 mg of testosterone enanthate) for 9 months. The T/LH ratio increased from a mean of 0.052 (range 0.002–0.108) at baseline to 45.16 (1.28–252) at 3 months, 85.7 (8.3–238) at 6 months, and 71.7 (5.3–344) at 9 months. The authors indicated that, among all the different ratios or proposed markers they investigated, the urinary T/LH ratio showed the most dramatic increase (~ 1000 -fold). Of the other markers, the increase in the serum T/LH ratio was of similar magnitude as that of the urinary T/LH ratio, whereas the urinary T/E ratio had only a 50-fold increase. The investigators also reported that 1 of the 11 subjects produced a T/E ratio below the IOC cutoff at 3 and 9 months of administration and just over the threshold at 6 months. The T/LH ratio for the same subject was above

the upper reference limit at 3, 6, and 9 months. Palonek et al. concluded that increased serum and urinary T/LH ratios in the presence of a normal T/E ratio may indicate self-administration of both T and E.

Unanswered is whether the T/LH ratio might be more sensitive than the T/E ratio for identifying illicit use of AS. Thus the goal of the present study was to determine which laboratory test is most sensitive and specific for detecting the administration of exogenous T.

Materials and Methods

SUBJECTS AND STUDY DESIGN

Healthy male volunteers between ages 18 and 40 years were recruited and, after an explanation of the study, gave their signed informed consent. The study protocol was reviewed and approved by the Human Subjects Institutional Review Board and the Clinic Research Center of the University of Iowa. A standard drug history, developed by the National Institute on Drug Abuse, was administered before entry. Any subject who indicated he was currently using central nervous system stimulants other than modest amounts of caffeine (two cups of coffee per day) was excluded from the study.

Each subject received two weekly placebo doses of cottonseed oil, the vehicle for testosterone cypionate (TC). At the end of the 2-week placebo lead-in period, subjects were randomized to one of three doses of TC (100, 250, or 500 mg/week) given for 14 consecutive weeks. In our experience with AS users [10], the subjects' shortest average cycle was 7 weeks, the longest 14 weeks. Thus, we decided that subjects should be administered TC for a typical 14-week AS cycle to mimic the maximum cycling interval.

Subjects functioned as their own controls. They received weekly intramuscular injections of either TC or placebo (vehicle only) for 28 consecutive weeks. For the purpose of evaluating the effectiveness of the urine T/E ratio as an indicator of recent AS use, we considered only the subjects receiving supraphysiological TC doses (250 and 500 mg/week). A 100 mg/week dose is generally regarded as a physiological replacement dose in the majority of patients.

To monitor the subjects medically, we assessed their liver-function tests, fasting lipid profiles, thyroxine-binding globulin, sex-hormone-binding globulin, 24-h urinary free cortisols, serum free and total T, estradiol, LH, follicle-stimulating hormone, thyroid-stimulating hormone, and free thyroxine—obtained at baseline, at entry into the study, after the 2-week placebo injection period, and then biweekly for the remainder of the study. All endocrine samples were collected between 0700 and 0900 to minimize the chronotropic secretion effects of these hormones. Depo[®]-testosterone (TC), 200 g/L (200 mg/mL), was the proprietary product utilized for the study. The diluent (0.2 mL of benzyl benzoate, 9.45 mg of benzyl alcohol, and 560 mg of cottonseed oil per milliliter) was prepared by the Pharmaceutical Services Division of the

University of Iowa College of Pharmacy (an FDA-approved manufacturing group). At the end of the 14 weeks of TC administration, the subjects were switched to diluent-only injections.

ASSAYS

The urine drug screens were performed by Smith Kline Beecham Clinical Laboratories Sports Testing Center in Tucker, GA, a laboratory certified by the US Department of Health and Human Services. The initial drug screen and all subsequent screens were negative for AS (other than T), amphetamines, barbiturates, benzodiazepines, cocaine metabolites, methadone, methaqualone, opiates, phenylclidine, and propoxyphene.

Urine concentrations of T, E, LH, and creatinine were also determined in the samples (assayed by Smith Kline Beecham). The urine samples were refrigerated at 8 °C and were analyzed within 5–10 days after collection. If the T/E ratio was <6, the sample was discarded within 30 days. Urine drug screens were routinely obtained at weeks 0, 1, 4, 8, 12, 16 or 17, 20, 24, and 28; in some follow-up cases, they were obtained at weeks 40 and 92. AS screens and confirmations were performed by GC-MS on separate aliquots. Samples were initially screened for the substance abuse panel by Emit (Behring, Palo Alto, CA); all positive results were confirmed by GC-MS [11]. LH in urine was performed by Microparticle Enzyme Immunoassay with the Abbott Diagnostics (Chicago, IL) IMx system.

The T/E ratio was determined by GC-MS. Both free and conjugated T and E were extracted with C₁₈ solid-phase extraction columns (Bond Elute LRC; Varian, Harbor City, CA), hydrolyzed with β -glucuronidase (Boehringer Mannheim, Mannheim, Germany), and detected by monitoring characteristic ions with the mass spectrometer. Quantification and identification of T and E required selected-ion mode analysis in which the presumptive positive specimens were matched with the retention times and ion ratios of known compounds. The T calibration curve was linear between 2 and 400 μ g/L; that for E was linear between 2 and 500 μ g/L. The CV for the T/E ratio was 13.3%. The specificity of this method is extremely high: At the time of the performance of the assays, no compounds were known to interfere with either T or E.

The urine concentration of LH was determined with the IMx system kit for serum LH as described in the 1991 IMx LH package insert. To determine that there was no matrix effect for the assay, we added known amounts LH to urine and serum samples and found that the resulting calibration curves could be superimposed on each other and were linear between 2 and 600 IU/L. The lower limit of detection for this assay is 0.5 IU/L. The CV for the serum LH assay is 8.7% at 5.37 IU/L, 6.4% at 43.2 IU/L, and 6.2% at 82.5 IU/L.

All serum samples for determining free and total T were stored at -20 °C until assay. The T concentrations were quantified with Coat-A-Count® kits (Diagnostic

Products Corp., Los Angeles, CA) as described in the manufacturer's package insert (1995). The lower limits of detection were 40 ng/L for total T and 0.15 ng/L for free T. The inter- and intraassay CVs for the free T assay were 11.2% and 5.5%, respectively; those for total T were 10.4% and 8.8%, respectively.

Results

In all, 93 urine drug screen samples were obtained from the 19 subjects participating in the study who received supraphysiological doses of TC. Seven received 250 mg/week and 12 took 500 mg/week. None of the subjects was positive for exogenous AS use other than for the TC injections administered during weeks 2–15 of the study. Concentrations of free T in serum were analyzed 5–7 times between days 3 and 21 after the last TC injection for 17 of the 19 subjects. From these data, we calculated for each patient the terminal elimination rate (k_e) and the elimination half-life ($t_{1/2}$) for free T in serum. To determine k_e , we fit the T concentrations $f(t)$ and time points (t) to the following single exponential decay equation, where a is the concentration of T at time 0:

$$f(t) = ae^{-k_e t} \quad (1)$$

We determined $t_{1/2}$ as follows:

$$t_{1/2} = 0.693/k_e \quad (2)$$

A 21-day T sampling period was appropriate to determine the $t_{1/2}$ of exogenous T because gonadotropin-releasing-hormone stimulation tests indicated that the hypothalamic-pituitary-testicular axes of the subjects did not regain sufficient sensitivity to stimulate release of T until 4–6 weeks after discontinuation of the TC injections.

Table 1. Elimination half-life of free testosterone in 19 subjects.

| Subject | TC dose, mg/week | Free T elimination half-life, days |
|---------|------------------|------------------------------------|
| 2455 | 250 | 8.2 |
| 2908 | 250 | 6.0 |
| 3058 | 250 | 6.4 |
| 3626 | 250 | 4.8 |
| 5361 | 250 | 6.4 |
| 9298 | 250 | 6.4 |
| 0030 | 250 | 14.1 |
| 1078 | 500 | 6.0 |
| 2166 | 500 | 5.0 |
| 3415 | 500 | 7.3 |
| 4008 | 500 | 7.8 |
| 4045 | 500 | 4.9 |
| 4249 | 500 | 4.8 |
| 5340 | 500 | 7.4 |
| 6218 | 500 | Not available |
| 6534 | 500 | 6.8 |
| 9338 | 500 | 5.5 |
| 0153 | 500 | 4.2 |
| 0012 | 500 | Not available |

The individual elimination half-life data are presented in Table 1. There was no difference in half-life values between the weekly TC doses of 250 and 500 mg (Mann-Whitney $U = 25.0$, $P = 0.37$). The overall mean \pm SD elimination half-life for free T in serum after administration of TC was 6.6 ± 2.3 days. Based on these data, an 11-day $t_{1/2}$ would be 2 SD from the mean. Given that 97% of the exogenous T was excreted in 5 half-lives (i.e., 11-day half-life \times 5 half-lives = 55 days, or 8 weeks) and that pituitary sensitivity to gonadotropin-releasing hormone returned within 4–6 weeks of the last TC injection, subjects were assumed to have ceased taking ("be off") exogenous T by the time of the urine drug screen performed 9 weeks after the last TC injection.

The AS urine drug screen findings indicated that the urinary T/E ratio cutoff of ≥ 6 , the traditional laboratory marker to determine the use of exogenous T and used as such by the National Collegiate Athletic Association and the IOC, although quite specific for determining nonuse of T, is not a sensitive indicator for detecting illicit T usage. Table 2 illustrates this. Although the T/E ratio of >6 had 96% specificity in identifying our subjects as being off steroids by 9 weeks after their last dose, it was correct only 54% of the time for identifying our subjects as being on steroids during the 14 weeks of TC injections and in the 9 subsequent weeks when they received sham injections. As a practical matter, these data suggest that one of every two subjects using injectable TC will, both during injection periods and for 9 weeks afterwards, give a false-negative urine drug screen. Receiver operating characteristic (ROC) analysis of these data [12] identified a urinary T/E ratio of ≥ 1.2 as the cutoff value that provided optimum sensitivity and specificity for indicating use or nonuse of T. Resorting the data in Table 2 illustrates that use of a T/E ratio of ≥ 1.2 for a T-positive urine improves the sensitivity to 83% and the specificity decreases only somewhat, to 77%.

The potential usefulness of the urine T/LH ratio as an indicator of T use and nonuse is illustrated in Table 3. These data suggest that to maintain 100% specificity requires a threshold T/LH ratio of ≥ 74 , although the sensitivity at this cutoff is only 52%. However, by ROC analysis (data not shown), the urinary T/LH ratio cut-

Table 3. Contingency table for urinary T/LH ratios used as the threshold ratio for anabolic steroid use (TC 250 or 500 mg/week).

| T/LH ratio | No. of subjects | |
|-------------|-----------------|---------------------|
| | On TC | Off TC ^a |
| $\geq 74^b$ | 24 | 0 |
| $< 74^b$ | 22 | 47 |
| $\geq 30^c$ | 35 | 6 |
| $< 30^c$ | 11 | 41 |

^a Subjects who tested negative 9 weeks after their last TC injection.

^b $\chi^2 = 33.051$, $P < 0.0001$, sensitivity = 52%, specificity = 100%.

^c $\chi^2 = 37.814$, $P < 0.0001$, sensitivity = 76%, and specificity = 87%.

point that optimizes sensitivity and specificity is ≥ 30 . As Table 3 shows, use of a urinary T/LH ratio ≥ 30 increases sensitivity to 76% but decreases specificity to 87%.

Discussion

From a medical-legal standpoint the most worrisome finding of these data is the false-positive tests. Table 4 characterizes the false positives—i.e., a test result that is not negative at 9 weeks after the last TC injection—for the various testing schemes. Nine weeks is equivalent to the amount of time required to clear 97% of the exogenous TC. For the urinary T/E ratio of 6, only two subjects did not meet this criteria, whereas for the urine T/LH ratio of 30, four subjects did not meet this criteria. All six patients who tested "positive" were actually tested 9–25 weeks after their last TC injection. When contrasted with the half-life data, this suggests that the normalization of LH concentrations may lag behind the rate at which the exogenous T clears from the body. Moreover, in reality there are no truly false-positive test results. However, in no case did a subject's urine screen test positive before the start of the TC injections.

The mean \pm SD urinary T/LH and T/E ratios before the start of the TC injections were 3.8 ± 2.4 and 0.8 ± 1.3 , respectively. Other than when the subjects were receiving T injections, the only time there was a significant difference between pre-TC injection urinary T/E and T/LH ratios and the ratios after the start of the TC injections was 2 weeks after the last injection. For the urinary T/LH ratio, the mean difference between the baseline value and the ratio 2 weeks after the last injection was 29.8 ($t = 2.829$, $P < 0.02$, $df = 16$); for the urine T/E ratio, the mean difference was 14.9 ($t = 2.703$, $P < 0.02$, $df = 16$).

As suggested in Table 4, the urinary T/LH ratio of ≥ 30 is the screen most likely to detect AS use the longest, i.e., as long as 25 weeks after the last injection. Using the urinary T/LH ratio ≥ 30 as a marker showed that 4 of 19 (21%) subjects tested positive 9–25 weeks after their last injection of T. In contrast, use of the urinary T/E ratio ≥ 6 found only 2 of 19 (11%) patients positive for steroid usage at 9 weeks after their last T injection. Fig. 1 chronologically contrasts the mean urine T/LH and T/E ratios. Inspection of Fig. 1 suggests that the urinary T/LH

Table 2. Contingency table for various urinary T/E ratios used as the threshold ratio for anabolic steroid use (TC 250 or 500 mg/week).

| T/E ratio | No. of subjects | |
|--------------|-----------------|---------------------|
| | On TC | Off TC ^a |
| $\geq 6^b$ | 25 | 2 |
| $< 6^b$ | 21 | 45 |
| $\geq 1.2^c$ | 38 | 11 |
| $< 1.2^c$ | 8 | 36 |

^a Subjects who tested negative 9 weeks after their last TC injection.

^b $\chi^2 = 28.312$, $P < 0.0001$, sensitivity = 54%, specificity = 96%.

^c $\chi^2 = 32.689$, $P < 0.0001$, sensitivity = 83%, specificity = 77%.

Table 4. False-positive rates for use of supraphysiological doses of anabolic steroids in different urine testing schemes.

| Test cutoff | False-positive rates | | Subject description | | |
|----------------|----------------------|-----------------|---------------------|------------------------------|----------------------|
| | % | No. of subjects | TC dose, mg/week | No. of weeks since last inj. | Ratio |
| T/E ≥ 6 | 4 | 2 | 500 | 9 | 7.3 |
| | | | 500 | 9 | 6.5 |
| T/LH ≥ 30 | 13 | 4 | 500 | 9 | 73 |
| | | | 500 | 9 & 13 ^a | 65 & 35 ^a |
| | | | 250 | 13 | 64 |
| | | | 250 | 13 & 25 ^a | 47 & 34 ^a |

^a Same subject, two times.

ratio returns to baseline at a slower rate than the urinary T/E ratio does, thereby explaining the greater number of false-positive results for T/LH in this group. To prove this point, we regressed the mean T/E and T/LH ratios against their timepoints at weeks 17, 20, 24, 28, and 40 and fit this as a monoexponential decay curve. The regression line intersects the critical T/LH ratio of 30 at 7.9 weeks after the last TC injection (T/LH ratio = $90.2 e^{-0.14(\text{week})}$, $r^2 = 0.86$). However, the T/E ratio fitted to the exponential equation (T/E ratio = $12.3 e^{-0.1916(\text{week})}$, $r^2 = 0.80$) intersects the ratio of 6 at 3.7 weeks. Both models, therefore, demonstrate why more subjects test positive for a longer time when assessed with the T/LH ratio.

It is not uncommon for nonpower athletes (e.g., distance runners, swimmers, tennis players, soccer players) to utilize physiological doses of T (i.e., TC 100 mg/week) to counter the catabolic effects of stress and exercise on muscle. We measured urine T/E and urine T/LH in seven subjects who were administered TC at 100 mg/week. Monoexponential regression equations for the T/E and T/LH ratios to return to baseline values were based on the mean ratios measured in these subjects at weeks 17, 20, and 24 after cessation of TC injections. The urinary T/E ratio, when fitted as an exponential decay equation

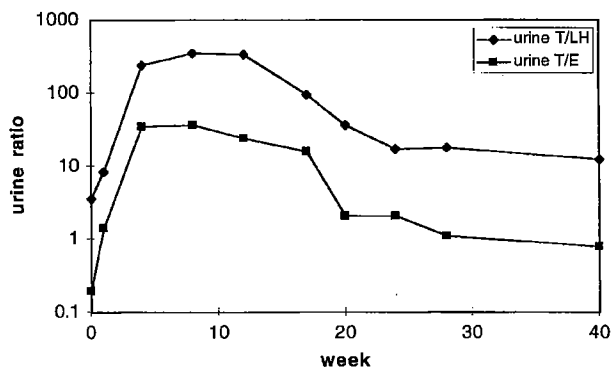


Fig. 1. Urine T/LH ratios and T/E ratios in 19 subjects receiving TC, 250 or 500 mg/week.

(T/E ratio = $8.3 e^{-0.2072(\text{week})}$, $r^2 = 0.82$), intersects the ratio of 6 at 1.6 weeks, whereas the urinary T/LH ratio, fitted to the equation T/LH ratio = $48.9 e^{-0.2148(\text{week})}$ ($r^2 = 0.99$), intersects the ratio of 30 at 2.3 weeks. These data suggest that is debatable whether TC at 100 mg/week is actually a physiological replacement dose: Some athletes may test positive even at this small a dose of T.

In conclusion, we find that the urinary T/LH ratio is a more sensitive and specific test for a longer time for investigating AS use than is the urinary T/E ratio. Supporting this finding is the fact that, unlike the case for E, there are no commercially available FDA-approved LH products. This advantage alone makes the urinary T/LH ratio a considerably more practical screening test than the urinary T/E ratio.

This project was supported by a grant from the National Institute on Drug Abuse at the National Institutes of Health (RO1DA08347).

References

- Kicman A, Oftebro H, Walker C, Norman N, Cowan DA. Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes. *Clin Chem* 1993;39:1798-803.
- Matsumoto AM. Effects of chronic testosterone administration in normal men: safety and efficacy of high-dosage testosterone and parallel dose-dependent suppression of luteinizing hormone, follicle-stimulating hormone, and sperm production. *J Clin Endocrinol Metab* 1990;70:282-7.
- Palonek E, Gottlieb C, Garle M, Björkhem I, Carlstrom K. Serum and urinary markers of exogenous testosterone administration. *J Steroid Biochem Mol Biol* 1995;55:121-7.
- Dehennin L. Detection of simultaneous self-administration of testosterone and epitestosterone in healthy men. *Clin Chem* 1994;40:106-9.
- Catlin DH, Hatton CK. Use and abuse of anabolic and other drugs for athletic enhancement. *Adv Intern Med* 1991;36:399-424.
- Dehennin L, Matsumoto A. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. *J Steroid Biochem Mol Biol* 1993;44:179-89.
- Dehennin L. On the origin of physiologically high ratios of urinary testosterone to epitestosterone: consequences for reliable detection of testosterone administration by male athletes. *J Endocrinol* 1994;142:353-60.
- Brooks R, Jeremiah G, Webb W, Wheeler M. Detection of anabolic steroid administration to athletes. *J Steroid Biochem* 1979;11:913-7.
- Kicman A, Brooks R, Collyer S, Cowan DA, Nanjee MN, Southan GJ, Wheeler MJ. Criteria to indicate testosterone administration. *Br J Sports Med* 1990;24:253-64.
- Perry PJ, Andersen KH, Yates WR. Illicit anabolic steroid use in athletes: a case series analysis. *Am J Sports Med* 1990;18:422-8.
- Sample RHB, Baenziger JC. Gas chromatography/mass spectrometric detection of anabolic steroids. In: Deutsch J, ed. *Analytical aspects of toxicology*. New York: JR Wiley and Sons, 1989:247-72.
- Somoza E, Mossman D. Neuropsychiatric decision making: designing nonbinary diagnostic tests. *J Neuropsychiatry Clin Neurosci* 1991;3:197-200.

A Single Dose of the Potent Gonadotropin-Releasing Hormone Antagonist Acyline Suppresses Gonadotropins and Testosterone for 2 Weeks in Healthy Young Men

KAREN L. HERBST, ANDREA D. COVIELLO, STEPHANIE PAGE, JOHN K. AMORY, BRADLEY D. ANAWALT, AND WILLIAM J. BREMNER

Department of Medicine, Division of Endocrinology, Charles R. Drew University (K.L.H.), Los Angeles, California 90059; Center for Research in Reproduction and Contraception, Department of Medicine, University of Washington (A.D.C., S.P., J.K.A., B.D.A., W.J.B.), and Medical Service, Department of Veteran Affairs, Puget Sound Health Care System (B.D.A.), Seattle, Washington 98195

Acyline is a novel GnRH antagonist that reliably inhibits gonadotropins and testosterone (T) levels in men for 48 h after a single dose up to 75 $\mu\text{g/kg}$. In this study we examined gonadotropin and T levels in 28 healthy young men administered acyline as single doses of 150 or 300 $\mu\text{g/kg}$ or serial injections of 75 $\mu\text{g/kg}$. A single 300 $\mu\text{g/kg}$ dose of acyline suppressed gonadotropins and T to castrate levels for 15 d (baseline, 21.1 ± 3.1 ; nadir, 1.95 ± 0.4 nmol/liter; mean \pm SEM; $P < 0.05$). Serum acyline levels peaked 90 min after the injection of 300 $\mu\text{g/kg}$ acyline to a maximum concentration of 112.4 ± 18 ng/ml ($n = 7$; $t_{1/2} = 4.9$ d). Injections of 75 $\mu\text{g/kg}$ acyline every 2 d for five

doses suppressed gonadotropins for more than 20 d (nadir T, 1.06 ± 0.17 nmol/liter; $P < 0.05$ compared with baseline). Adverse events were mild and included erythema and pruritus at the injection site. Acyline, therefore, is one of the most potent peptide GnRH antagonists studied to date with minimal adverse events. A twice monthly injection of acyline could be used as a potent suppressor of the GnRH axis to advance the development of a hormonal male contraceptive or for treatment of hormonally dependent disease. (*J Clin Endocrinol Metab* 89: 5959–5965, 2004)

GnRH IS A hypothalamic decapeptide that is synthesized and released in a pulsatile manner from hypothalamic neurosecretory cells and regulates the synthesis and release of pituitary gonadotropins that, in turn, regulate steroidogenic and gametogenic functions of the gonads. GnRH plays a rate-limiting role in reproductive processes, and thousands of GnRH analogs have been generated because of their potential clinical utility. Most GnRH analogs are employed in the treatment of prostate cancer and for their utility in the disruption of LH surges for the regulation of ovulation induction in women undergoing *in vitro* fertilization (1–3). Other clinical uses for GnRH analogs include endometriosis and other gynecological diseases (4, 5), precocious puberty (6), and hormonal male contraception (7, 8). Both GnRH agonists and antagonists suppress gonadotropins and gonadal steroids, but the administration of GnRH agonists is accompanied by an initial gonadotropin and gonadal hormone surge known as a flare, delaying suppression by 7–14 d (2). GnRH antagonists do not cause flare, because they competitively block and inhibit GnRH-induced GnRH receptor gene expression, leading to immediate pituitary suppression.

Acyline is a GnRH antagonist sponsored by the NICHD that reliably maintained suppression of gonadotropins and testosterone (T) for 48 h with doses up to 75 $\mu\text{g/kg}$ by sc injection (9). We sought to determine whether larger or serial

doses of acyline would suppress gonadotropins and T for sufficiently long periods of time to allow a depot formation of acyline to be conveniently used for clinical applications such as a male hormonal contraception.

We administered acyline as single doses of 150 and 300 $\mu\text{g/kg}$ and as serial injections of 75 $\mu\text{g/kg}$ and monitored serum gonadotropin and T levels for a period of 30 d or until serum gonadotropins and T returned to baseline levels.

Subjects and Methods

Acyline

Acyline was originally synthesized by Jean Rivier at The Salk Institute (La Jolla, CA) (10) and is distributed by the NICHD. Acyline is prepared as a lyophilized powder (4.4 mg/vial) and is stored at -20°C . Acyline-lyophilized powder was suspended in bacteriostatic water to a final concentration of 2 mg/ml for injection into sc abdominal tissue. Serum levels of acyline were measured by RIA in a subset of subjects after the injection of 300 $\mu\text{g/kg}$ acyline, using a specific antiserum and a proprietary peptide with authentic peptide standard (Woods Assay, Inc., Portland, OR) as described previously (11). The sensitivity of the assay for acyline was 0.35 ng/ml.

Subjects

All study procedures involving human subjects were approved by the institutional review board at University of Washington and were performed at the University of Washington Clinical Research Center in accordance with institutional guidelines. Thirty-five men (age, 18–50 yr) were recruited by flyers posted on local college campus bulletin boards. All subjects were healthy, eugonadal men with normal medical histories and baseline physical examinations, including normal testicular size by Prader orchidometer and prostate size by digital rectal exam, serum chemistries, complete blood count, and gonadotropin and T levels. We excluded subjects who were current smokers, drank more than 7 oz

Abbreviations: ACY 150, 150 $\mu\text{g/kg}$ Acyline; T, testosterone.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

alcohol weekly, or had taken prescription medications or any sex steroid hormone within the last 6 months. Of these men, 34 were screened for study eligibility. Six men were excluded or withdrawn from the study after the screening process and before drug treatment: one man had low T levels, one man had abnormal hematology results at screening, one man had an elevated alanine aminotransferase test value, and two men had time constraints that prevented study participation. One subject was screened but did not begin the drug treatment phase, because recruitment for the study was complete.

Subjects were assigned to one of four groups ($n = 7$): group 1, single dose of 150 $\mu\text{g/kg}$ acyline on d 0 (ACY 150); group 2, single dose of 300 $\mu\text{g/kg}$ acyline on d 0 (ACY 300); group 3, 75 $\mu\text{g/kg}$ acyline injections on d 0, 4, and 8 and placebo on d 2 and 6 (ACY 75 \times 3); and group 4, 75 $\mu\text{g/kg}$ acyline injections on d 0, 2, 4, 6, and 8 (ACY 75 \times 5).

Acyline was administered by sc injection in the abdomen between 0700 and 1000 h. For ACY 150 and 300 groups, blood samples were obtained at 30, 60, 90, and 120 min; 3, 4, 6, 8, 12, and 24 h; and 2, 3, 4, 7, 9, 11, 13, 15, 17, 19, 21, 25, and 30 d after injection. For the serial injection groups ACY 75 \times 3 and 75 \times 5, blood samples were obtained on d 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 21, 25 and 30. All research subjects were monitored for 30 d with vital signs determined, and laboratory tests and physical exams performed.

Measurements

Hormones. FSH, LH, and T levels were measured by immunofluorometric assay (Delfia, Wallac Oy, Turku, Finland). Samples from a given individual were measured in a single assay. The sensitivities of the assay for FSH and LH were 0.016 and 0.019 IU/liter, respectively. For low, mid, and high pooled values of 0.054, 1.04, and 20.8 IU/liter FSH, the intraassay coefficients of variation were 12%, 1.9%, and 2.9%, and the interassay coefficients of variation were 18%, 6.1%, and 4.1%, respectively. For low, mid, and high pooled values of 0.056, 0.95, and 15.6 IU/liter LH, the intraassay coefficients of variation were 6.5%, 3.9%, and 5.4%, and the interassay coefficients of variation were 21%, 8%, and 6.6%, respectively. The assay sensitivity for T was 0.5 nmol/liter. For low, mid, and high pooled values of 3.8, 10.6, and 24.4 nmol/liter T, the intraassay coefficients of variation were 9.6%, 5.2%, and 6.1%, and the interassay coefficients of variation were 12%, 8.2%, and 6.7%, respectively. If serum T levels had not returned to baseline within 30 d after the injection of acyline, subjects were requested to return for additional blood sampling until serum T levels had normalized.

Serum laboratory tests. Screening and monitoring laboratory tests for complete blood count, electrolytes, glucose (chemistry 7), calcium, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, bilirubin, and total protein were performed at the University of Washington.

Statistics

FSH, LH, and T are expressed as the mean hormone level \pm SEM. For statistical analysis, all hormone data were log-transformed and then back-transformed for ease of presentation. Comparisons of data within groups and between groups were analyzed with ANOVA (SigmaStat, SPSS, Inc., Chicago, IL). Evidence for change from baseline within a group was further analyzed at each time point by paired t tests. The area under the curve was calculated using the trapezoid rule from time zero to the last measured level without smoothing or curve fitting (PK Solutions, Summit Research, Montrose, CO). The half-life ($t_{1/2}$) was calculated from 3 d to the last measured level. For all comparisons, an α of 0.05 was considered significant.

Results

Study population

The 28 subjects enrolled in this study were eugonadal and lean by body mass index (Table 1). There were no significant differences in baseline parameters between groups.

Acyline administration

Acyline was initially suspended in bacteriostatic water at a concentration of 8.8 mg/ml, which allowed acyline to be administered as a single, small volume injection at a concentration of 300 $\mu\text{g/kg}$ (2.4-ml injection for a 70-kg man). Nodule formation at the sites of acyline injection (lingering for as long as 30 d postinjection) suggested that the drug might be forming a gel in the fat tissue in a volume large enough to be palpable, similar to other GnRH antagonists (12). Suppression of gonadotropins and T was also inconsistent (data not shown). Acyline was therefore administered as a 2.0 mg/ml solution in water that does not lead to palpable nodule formation when injected into sc tissue (9), but did require multiple injections for a dose of 150 or 300 $\mu\text{g/kg}$. A 70-kg man administered 300 $\mu\text{g/kg}$ acyline at a concentration of 2.0 mg/ml would have an sc injection of 10.5 ml in four or five divided doses (2–3 ml/injection).

Single injections of acyline

Gonadotropins. Baseline levels of FSH and LH are shown in Table 1. Both FSH and LH decreased rapidly after a single dose of 150 or 300 $\mu\text{g/kg}$ acyline in all subjects (Fig. 1). In the ACY 150 group, FSH levels dropped significantly below baseline 8 h after injection, reaching a nadir at 3 d (1.06 ± 0.35 IU/liter), and remained significantly below baseline for 4 d after injection. LH levels decreased significantly below baseline by 1.5 h after injection, reached a nadir at 2 d (0.27 ± 0.05 IU/liter), and remained significantly below baseline for 3 d after injection.

In the ACY 300 group, FSH levels decreased significantly below baseline 1 h after injection and remained at these levels for 21 d, reaching a nadir on d 11 (0.26 ± 0.04 IU/liter). LH levels decreased significantly below baseline by 1.5 h after injection and reached a nadir at 2 d (0.17 ± 0.1 IU/liter), remaining significantly below baseline levels for 15 d. Gonadotropin levels tended to rebound above baseline by d 30 after the injection of acyline, but there was no significant difference between baseline and d 30 FSH or LH levels in the ACY 150 or ACY 300 groups.

T. Baseline levels of T for ACY 150 and ACY 300 are shown in Table 1. T rapidly decreased significantly below baseline

TABLE 1. Baseline parameters of subjects

| Group | ACY 150 | ACY 300 | ACY 75 \times 3 | ACY 75 \times 5 |
|--------------------------------------|----------------|----------------|-------------------|-------------------|
| n | 7 | 7 | 7 | 7 |
| Age (yr) | 26.6 \pm 2.7 | 31.9 \pm 3.8 | 30.9 \pm 2.1 | 23.0 \pm 1.8 |
| Body mass index (kg/m ²) | 26.1 \pm 1.7 | 24.5 \pm 0.7 | 25.6 \pm 1.1 | 26.2 \pm 1.6 |
| FSH (IU/liter) | 2.5 \pm 0.3 | 2.7 \pm 0.8 | 3.1 \pm 0.5 | 2.0 \pm 0.3 |
| LH (IU/liter) | 3.2 \pm 0.5 | 3.3 \pm 0.7 | 3.5 \pm 0.2 | 4.0 \pm 0.4 |
| T (nmol/liter) | 21.1 \pm 3.1 | 21.6 \pm 4.2 | 20.0 \pm 2.6 | 26.1 \pm 3.3 |

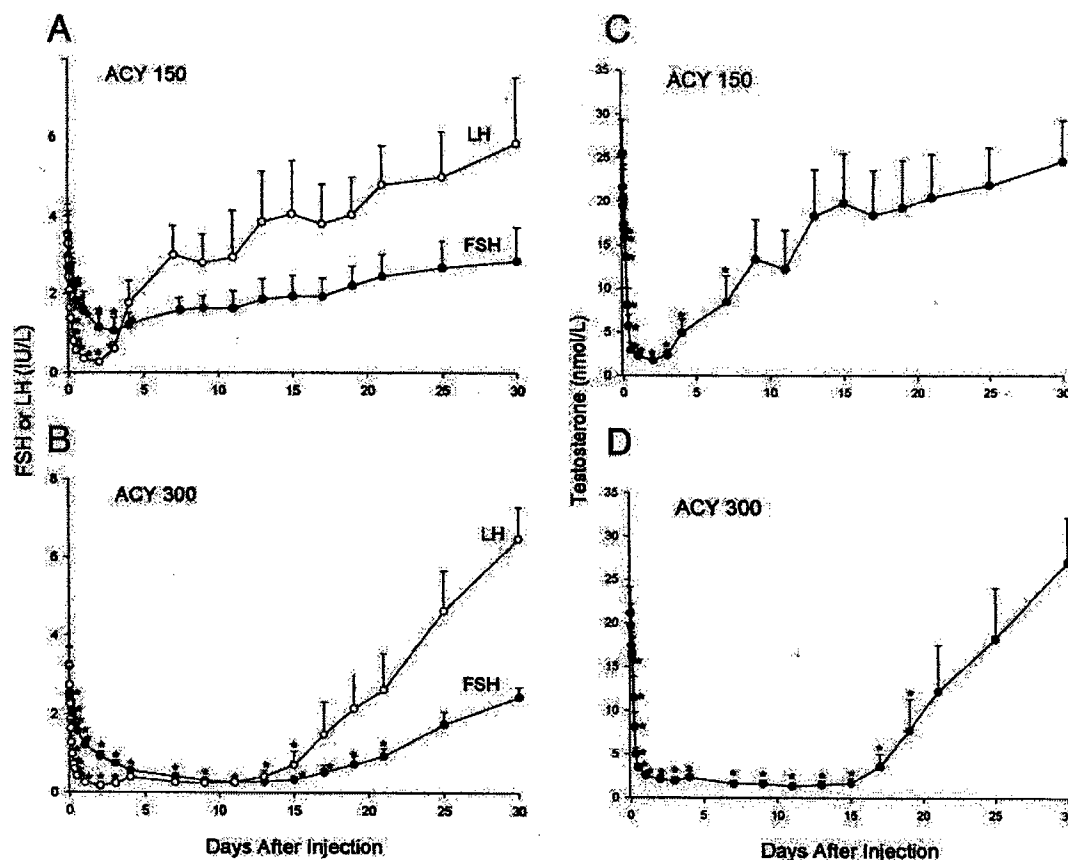


FIG. 1. Single injections of acyline rapidly suppress gonadotropins and T. Serum values of FSH (●) and LH (○) after 150 (A) and 300 (B) µg/kg acyline and serum values of T (●) after 150 (C) and 300 (D) µg/kg acyline are shown. Time is expressed as days after the first injection of acyline. Values (n = 7) are expressed as the mean ± SEM. *, $P < 0.05$ vs. baseline.

2 h after injection in all subjects after single acyline doses and decreased below castrate levels (5 nmol/liter) by 12 h after injection (Fig. 1). T levels reached a nadir in the ACY 150 group 2 d after injection (1.75 ± 0.28 nmol/liter) and began to rise but remained significantly below baseline through d 7 after injection. One subject's T level remained below baseline levels until d 37.

T levels reached a nadir on d 2 after injection in the ACY 300 group (1.95 ± 0.38 nmol/liter) and remained at approximately this same level through d 15. T levels began increasing on d 17 after injection, remaining significantly below baseline levels until d 19 after injection, but were not significantly different from baseline levels on d 21. One subject's T level remained below baseline levels until d 35. On the average, there was no significant difference between baseline and d 30 T levels in either group.

Multiple injections of acyline

Gonadotropins. In the ACY 75X3 group, FSH and LH levels decreased significantly below baseline on d 2 (Fig. 2, A and B). Gonadotropin levels increased before the next injection on d 4, but remained significantly below baseline, then decreased on d 6, reaching steady suppressed levels on d 10–18 after the third injection. The nadir level of FSH on d 18 was 0.39 ± 0.08 IU/liter, and the nadir level for LH was 0.16 ± 0.04 IU/liter on d 10. LH and FSH levels remained signifi-

cantly below baseline through d 21 and 25, respectively, but the levels normalized and were not significantly different from baseline levels by d 30.

In the ACY 75X5 group, LH and FSH decreased significantly below baseline by d 2 (Fig. 2, C and D). LH levels remained significantly suppressed below baseline from d 2–16, with a nadir level on d 10 (0.07 ± 0.01 IU/liter). FSH levels gradually decreased to a nadir on d 12 (0.1 ± 0.01 IU/liter) and remained significantly below baseline on d 2–25.

T. In the ACY 75X3 group, T decreased significantly below baseline levels after the first acyline injection (Fig. 3A), with small rebound increases before the second and third injections. T levels reached a nadir on d 10 after the third injection (1.29 ± 0.22 nmol/liter), remained significantly below baseline levels from d 2–21, and returned to baseline by d 30. Two subjects' T levels remained below baseline on d 30, but returned to baseline levels by d 33 and 47, respectively.

In the ACY 75X5 group, T levels decreased rapidly and significantly below baseline, reaching a nadir on d 17 (1.06 ± 0.17 nmol/liter) after the first injection and remained significantly suppressed through d 25, returning to baseline levels by d 30. Four subjects' T levels remained significantly below baseline levels on d 30, returning to baseline levels on d 36, 37, 45, and 47, respectively.

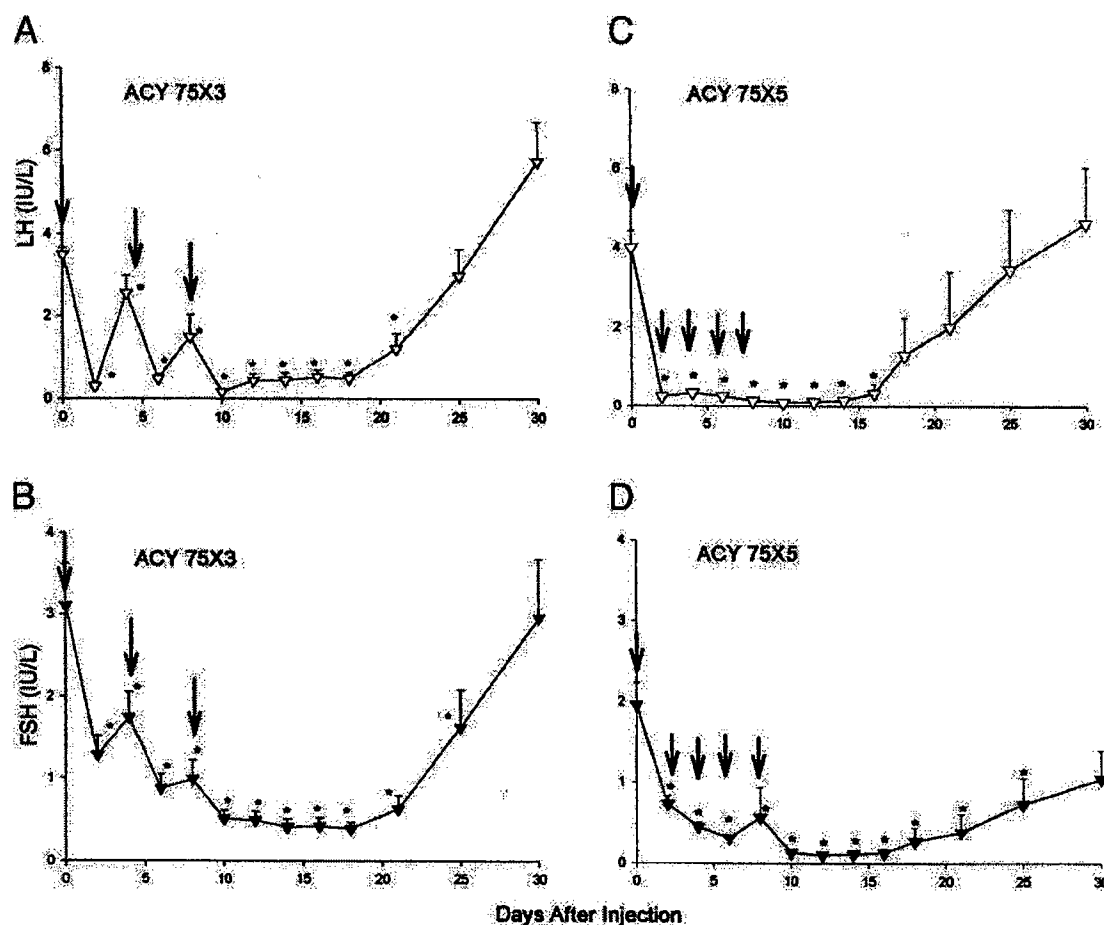


FIG. 2. Multiple injections of 75 µg/kg acyline can maintain suppression of gonadotropins for more than 20 d. Serum values of LH (A; Δ) and FSH (B; \blacktriangle) after three injections of 75 µg/kg acyline spaced 4 d apart (arrows) and LH (C) and FSH (D) levels after five injections of 75 µg/kg acyline spaced 2 d apart (arrows) are shown. Time is expressed as days after the first injection of acyline. Values ($n = 7$) are expressed as the mean \pm SEM. *, $P < 0.05$ vs. baseline.

Acyline pharmacokinetics

Serum acyline levels were measured in all seven subjects in the ACY 300 group (Fig. 4). Acyline reached a maximum concentration of 112.4 ± 6.9 ng/ml 90 min after injection and remained significantly elevated above background through d 30. Blood levels of acyline dropped by 50% at 4.9 d.

Adverse events and safety

Skin reactions were the most common side-effect noted after sc acyline injections. A mild pink blush occurred at the site of injection in 22 of 28 subjects (78.5%), lasting up to 120 min. The blush occurred in 94% of injections in subjects who experienced it. Pruritus at the site of injection occurred in 75% of subjects, with an average score of 2.3 ± 0.1 arbitrary units of 5: 0 = no itch, 1 = barely noticeable, 2 = mild, 3 = moderate, 4 = severe, and five = the worst itch ever experienced. The pruritus persisted for approximately 40 min on the average. Nodules or deep induration were noted in three individuals, one in each of the groups receiving single acyline doses (although multiple injections), and persisted at 2 and 11 d; in one subject receiving serial injections, they persisted for 2 d. Bruising at the site of injection was also noted in 12

individuals (42.9%), although not at all injection sites. During the hypogonadal period (T, <5 nmol/liter), 10 subjects experienced decreased libido or fatigue (35.7%), and two experienced hot flashes or significant changes in mood/irritability (7.1%). One subject experienced myalgia without signs of infection, including fever. There were no significant changes in any chemistry parameter during the study, including aspartate aminotransferase and alanine aminotransferase. The hematocrit decreased slightly in all 28 subjects from an average baseline value of $42.5 \pm 0.46\%$ to $41.1 \pm 0.49\%$ ($P < 0.01$), remaining in the normal range (38–50%). The white blood cell and platelet counts were unaffected by acyline administration.

Discussion

GnRH antagonists competitively block and inhibit GnRH-induced GnRH receptor gene expression, leading to immediate pituitary suppression (13) without the surge in gonadotropins and T seen after GnRH agonist administration (14). The GnRH antagonist, acyline, was developed to have greater potency and less histamine-mediated skin irritation than previous antagonists (10). A single injection of 300

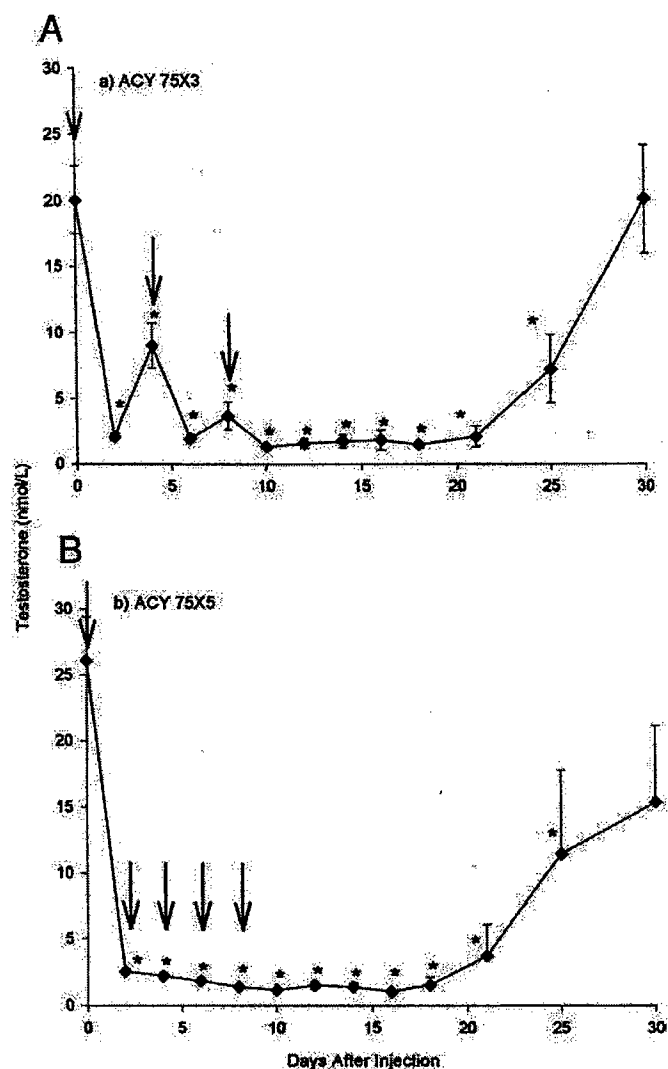


FIG. 3. Multiple injections of 75 $\mu\text{g/kg}$ acyline can maintain suppression of T for more than 20 d. Serum values of T (\diamond) after three injections of 75 $\mu\text{g/kg}$ acyline spaced 4 d apart (A, arrows) and five injections of 75 $\mu\text{g/kg}$ acyline spaced 2 d apart (B, arrows). Time is expressed as days after the first injection of acyline. Values ($n = 7$) are expressed as the mean \pm SEM. *, $P < 0.05$ vs. baseline.

$\mu\text{g/kg}$ acyline in this study rapidly and significantly suppressed gonadotropins and T to castrate levels (T, < 5 nmol/liter) for 15 d. Five injections of 75 $\mu\text{g/kg}$ acyline spaced 2 d apart also immediately suppressed gonadotropins and T and maintained T within the castrate range for up to 21 d. Five injections of 75 $\mu\text{g/kg}$ acyline might have suppressed the hypothalamic-pituitary-gonadal axis longer than the single 300 $\mu\text{g/kg}$ dose, because the cumulative dosage administered was greater at 375 $\mu\text{g/kg}$. Alternatively, because 75 $\mu\text{g/kg}$ is known to rapidly and effectively suppress gonadotropins and T for up to 48 h (9), serial injections of acyline every 2 d over an 8-d period might simply maintain that 48-h suppression. However, if the latter were true, then gonadotropins would be expected to begin returning to baseline approximately 48 h after the last injection. Instead, FSH and LH were still suppressed 8 d after the last of five injections of 75 $\mu\text{g/kg}$ acyline.

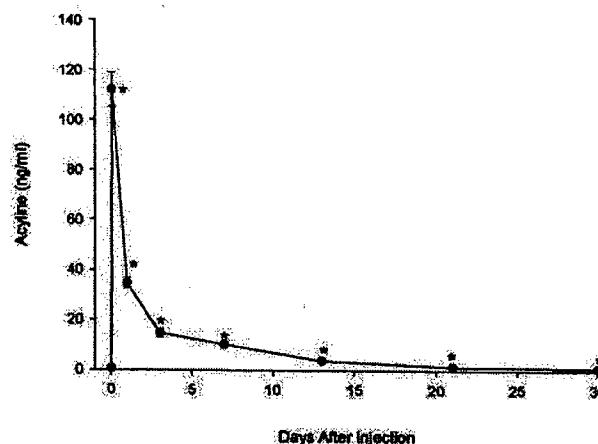


FIG. 4. Serum levels of acyline (nanograms per milliliter) remained significantly elevated for 30 d after sc injection of 300 $\mu\text{g/kg}$ acyline in seven healthy young men. Values are the mean \pm SEM after subtraction of 1 ng/ml background levels. *, $P < 0.05$ vs. baseline.

Cetrorelix is a GnRH antagonist that has similar potency to acyline (9, 15). Data examining the effect of cetrorelix on pituitary GnRH receptor expression and localization might help explain why serial injections of 75 $\mu\text{g/kg}$ acyline induce longer suppression of gonadotropins than a single 300 $\mu\text{g/kg}$ injection (15–18). GnRH receptors were significantly down-regulated for at least 72 h in rat pituitaries, accompanied by suppression of serum LH and T, after a single injection of 100 μg cetrorelix (16). The lowest receptor level was found 6 h after the injection of cetrorelix, but a marked recovery in receptor number was observed at 48 h. A major decrease in the expression of mRNA for pituitary LH-releasing hormone receptors was also found after chronic treatment with cetrorelix (17). This suppression is not believed to be a direct effect on gene expression of the GnRH receptor, but due to the fact that GnRH antagonists prevent up-regulation of receptor mRNA expression induced by GnRH (18). These data suggest that the prolonged suppression of gonadotropins and T by serial injections of 75 $\mu\text{g/kg}$ acyline is not only a reflection of the concentration of the antagonist at the level of the receptor, but that administration every 48 h might have prevented the up-regulation of GnRH receptor mRNA expression longer than the single dose of 300 $\mu\text{g/kg}$.

The question then becomes how is acyline best administered. Subjects and patients would probably prefer a single dose of sc acyline twice a month to serial injections. However, serial injections maintained suppression of gonadotropin and T levels longer than either single dose, as demonstrated by the four subjects whose T levels remained significantly below baseline more than 30 d after the start of the five injections. A minimal dose for a twice monthly injection might be 225 $\mu\text{g/kg}$, because gonadotropins and T were suppressed in the hypogonadal range for 14 d after three injections of 75 $\mu\text{g/kg}$ acyline. Alternatively, a higher dose injection than 300 $\mu\text{g/kg}$ might maintain acyline concentrations in the pituitary at a sufficiently high level to prevent up-regulation of GnRH receptor mRNA. The GnRH antagonist, abarelix, is administered as a 100-mg monthly injection to maintain suppression of serum gonadotropins and T (2). The amount of acyline administered to a 70-kg man in this

study as a 300 $\mu\text{g/kg}$ injection was 21 mg, approximately 5 times less than the dose of abarelix. It is therefore possible that a single higher dose of acyline might suppress gonadotropins and T for a month. The only drawback is the volume of acyline that would need to be administered (10.5 ml). A depot formulation of acyline is currently under development by the NICHD.

Adverse side-effects with acyline injection were again minimal, similar to our previous study (9), and included a blush at the injection site and mild pruritus. In this study, however, there was more bruising at the site of injection. There was no pattern to the bruising; it did not occur more commonly for specific individual subjects and was not associated more often with individual nurses who administered the injections. We believe that the bruising probably reflects differences in the manner the injection was administered, rather than being a result of the acyline itself. Three sc nodules at the site of injection were noted in this study: two lasting for 2 d, and one lasting 11 d. Because nodule formation did not occur with every injection in these individuals, these nodules probably represent a tissue reaction to the injection, rather than a reaction to acyline itself.

Other adverse events that occurred during this study in the hypogonadal period were expected as a result of declining T levels. These included hot flashes, decreased libido, fatigue, and irritability, consistent with symptoms of male hypogonadism (19, 20). Because T is known to increase the production of erythropoietin (21, 22), and castration decreases hemoglobin levels (23), our data demonstrating a small, but significant, decrease in hematocrit within the normal range was predictable.

The amount of time for acyline levels in serum to decrease by half ($t_{1/2}$) in this study was 4.9 d, greater in length than the 28.3 h previously found (9). This calculated $t_{1/2}$ for acyline in serum does not fit the classical definition of a true $t_{1/2}$, because it reflects not only the time required for half the total amount of acyline to be cleared from the serum, but also the rate of entry of acyline into serum from the presumed sc depot. Nevertheless, this calculated $t_{1/2}$ allows us to compare data from different studies. The difference found in the $t_{1/2}$ values between the two studies probably reflects the increased number of subjects tested for acyline levels in this study ($n = 7$) vs. the former study ($n = 4$), the similarity in suppression of gonadotropins in the current study (only two of four subjects had suppression of gonadotropins and T for 7 d in the previous study), and the higher dosage of acyline administered in the current study. The long calculated $t_{1/2}$ of acyline might also represent the ability of acyline to bind to serum proteins, as previously discussed (9), or a prolonged time of entry from the sc tissue into the serum compartment secondary to increased volume of injections.

Conclusion

Acyline is a likely candidate for use as a potent, long-lasting GnRH antagonist in the development of an effective male hormonal contraceptive regimen or for the treatment of sex steroid hormone-dependent syndromes, such as advanced severe endometriosis and prostate cancer. It is safe and potentially can be administered as a single dose, twice

a month. Because exogenous long-acting T formulations also inhibit circulating gonadotropins levels and spermatogenesis, a combination of long-acting acyline plus T might make a male hormonal contraceptive regimen a safe, effective, and practical option.

Acknowledgments

We thank Dorothy McGuiness and Arlen Sarkissian for their excellent technical support, and Connie Nosbisch for her efficient coordination of this study.

Received December 11, 2003. Accepted September 1, 2004.

Address all correspondence and requests for reprints to: Dr. Karen L. Herbst, Department of Medicine, Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R. Drew University, Room 3069 Third Floor, 1731 East 120th Street, Los Angeles, California 90059. E-mail: kaherbst@cdrewu.edu.

This work was supported by NIDDK Metabolism Training Grant T32-DK-O7247 (to K.L.H.), the NICHD/NIH through Cooperative Agreement U54-HD-12629 as part of the Specialized Cooperative Centers Program in Reproduction Research and through Cooperative Agreement U54-HD-42454 as part of the Cooperative Contraceptive Research Centers Program. A portion of this work was conducted through the Clinical Research Center facility at the University of Washington and was supported by NIH Grant M01-RR-00037.

References

- Huime JA, Lambalk CB 2001 Gonadotropin-releasing-hormone-receptor antagonists. *Lancet* 358:1793–1803
- Trachtenberg J, Gittleman M, Steidle C, Barzell W, Friedel W, Pessis D, Fotheringham N, Campion M, Garnick MB; Abarelix Study Group 2002 A phase 3, multicenter, open label, randomized study of abarelix versus leuprolide plus daily antiandrogen in men with prostate cancer. *J Urol* 167:1670–1674
- Tarlatzis BC, Bili HN 2003 Gonadotropin-releasing hormone antagonists: impact of IVF practice and potential non-assisted reproductive technology applications. *Curr Opin Obstet Gynecol* 15:259–264
- Felberbaum RE, Kupker W, Diedrich K 2002 Will GnRH antagonists assist in the treatment of benign gynaecological diseases? *Reprod Biomed Online* 5(Suppl 1):68–72
- Kupker W, Felberbaum RE, Krapp M, Schill T, Malik E, Diedrich K 2002 Use of GnRH antagonists in the treatment of endometriosis. *Reprod Biomed Online* 5:12–16
- Roth C 2002 Therapeutic potential of GnRH antagonists in the treatment of precocious puberty. *Expert Opin Investig Drugs* 11:1253–1259
- Wang C, Swerdloff RS 2004 Male hormonal contraception. *Am J Obstet Gynecol* 190:S60–S68
- Anawalt BD, Amory JK 2001 Advances in male hormonal contraception. *Ann Med* 33:587–595
- Herbst KL, Anawalt BD, Amory JK, Bremner WJ 2002 Acyline: the first study in humans of a potent, new gonadotropin-releasing hormone antagonist. *J Clin Endocrinol Metab* 87:3215–3220
- Rivier JE, Jiang G, Porter J, Hoeger CA, Craig AG, Corrigan A, Vale W, Rivier CL 1995 Gonadotropin-releasing hormone antagonists: novel members of the azaline B family. *J Med Chem* 38:2649–2662
- Goldman JM, Cooper RL, Rehnberg GL, Gabel S, McElroy WK, Hein J, Conn PM 1987 Age-related alterations in the stimulated release in vitro of catecholamines and luteinizing hormone-releasing hormone from the male rat hypothalamus. *Neurochem Res* 12:651–657
- Jiang G, Gavini E, Dani BA, Murty SB, Schrier B, Thanoo BC, DeLuca PP 2002 Identification and determination of GnRH antagonist gelling at injection site. *Int J Pharm* 233:19–27
- Kovacs M, Schally AV, Csemes B, Rekasi Z 2001 Luteinizing hormone-releasing hormone (LH-RH) antagonist cetorelix down-regulates the mRNA expression of pituitary receptors for LH-RH by counteracting the stimulatory effect of endogenous LH-RH. *Proc Natl Acad Sci USA* 98:1829–1834
- Tomera K, Gleason D, Gittleman M, Moseley W, Zinner N, Murdoch M, Menon M, Campion M, Garnick MB 2001 The gonadotropin-releasing hormone antagonist abarelix depot versus luteinizing hormone releasing hormone agonists leuprolide or goserelin: initial results of endocrinological and biochemical efficacies in patients with prostate cancer. *J Urol* 165:1585–1589
- Behre HM, Klein B, Steinmeyer E, McGregor GP, Voigt K, Nieschlag E 1992 Effective suppression of luteinizing hormone and testosterone by single doses of the new gonadotropin-releasing hormone antagonist cetorelix (SB-75) in normal men. *J Clin Endocrinol Metab* 75:393–398
- Halmos G, Schally AV, Pinski J, Vellido-Buenfil M, Groot K 1996 Down-

- regulation of pituitary receptors for luteinizing hormone-releasing hormone (LH-RH) in rats by LH-RH antagonist cetrorelix. *Proc Natl Acad Sci USA* 93: 2398–2402
17. Pinski J, Lamharzi N, Halmos G, Groot K, Jungwirth A, Vadillo-Buenfil M, Kakar SS, Schally AV 1996 Chronic administration of the luteinizing hormone-releasing hormone (LHRH) antagonist cetrorelix decreases gonadotrope responsiveness and pituitary LHRH receptor messenger ribonucleic acid levels in rats. *Endocrinology* 137:3430–3436
 18. Kovacs M, Schally AV 2001 Comparison of mechanisms of action of luteinizing hormone-releasing hormone (LHRH) antagonist cetrorelix and LHRH agonist triptorelin on the gene expression of pituitary LHRH receptors in rats. *Proc Natl Acad Sci USA* 98:12197–12202
 19. Seidman SN 2001 Testosterone deficiency and depression in aging men: pathogenic and therapeutic implications. *J Gend Specif Med* 4:44–48
 20. Zitzmann M, Nieschlag E 2000 Hormone substitution in male hypogonadism. *Mol Cell Endocrinol* 161:73–88
 21. Fisher JW, Langston JW 1968 Effects of testosterone, cobalt and hypoxia on erythropoietin production in the isolated perfused dog kidney. *Ann NY Acad Sci* 149:75–87
 22. Naets JP, Wittek M 1968 The mechanism of action of androgens on erythropoiesis. *Ann NY Acad Sci* 149:366–376
 23. Weber JP, Walsh PC, Peters CA, Spivak JL 1991 Effect of reversible androgen deprivation on hemoglobin and serum immunoreactive erythropoietin in men. *Am J Hematol* 36:190–194

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

Testosterone administration to older men improves muscle function: molecular and physiological mechanisms

ARNY A. FERRANDO,¹ MELINDA SHEFFIELD-MOORE,¹ CATHERINE W. YECKEL,¹
CHARLES GILKISON,² JIE JIANG,² ALISON ACHACOSA,³ STEVEN A. LIEBERMAN,²
KEVIN TIPTON,¹ ROBERT R. WOLFE,¹ AND RANDALL J. URBAN²
*Departments of ¹Surgery, ²Internal Medicine, and ³Physical Therapy,
The University of Texas Medical Branch, Galveston, Texas 77550*

Received 25 April 2001; accepted in final form 2 November 2001

Ferrando, Arny A., Melinda Sheffield-Moore, Catherine W. Yeckel, Charles Gilkison, Jie Jiang, Alison Achacosa, Steven A. Lieberman, Kevin Tipton, Robert R. Wolfe, and Randall J. Urban. Testosterone administration to older men improves muscle function: molecular and physiological mechanisms. *Am J Physiol Endocrinol Metab* 282: E601–E607, 2002. First published November 13; 10.1152/ajpendo.00362.2001.—We investigated the effects of 6 mo of near-physiological testosterone administration to older men on skeletal muscle function and muscle protein metabolism. Twelve older men (≥ 60 yr) with serum total testosterone concentrations < 17 nmol/l (480 ng/dl) were randomly assigned in double-blind manner to receive either placebo ($n = 5$) or testosterone enanthate (TE; $n = 7$) injections. Weekly intramuscular injections were given for the 1st mo to establish increased blood testosterone concentrations at 1 mo and then changed to biweekly injections until the 6-mo time point. TE doses were adjusted to maintain nadir serum testosterone concentrations between 17 and 28 nmol/l. Lean body mass (LBM), muscle volume, prostate size, and urinary flow were measured at baseline and at 6 mo. Protein expression of androgen receptor (AR) and insulin-like growth factor I, along with muscle strength and muscle protein metabolism, were measured at baseline and at 1 and 6 mo of treatment. Hematological parameters were followed monthly throughout the study. Older men receiving testosterone increased total and leg LBM, muscle volume, and leg and arm muscle strength after 6 mo. LBM accretion resulted from an increase in muscle protein net balance, due to a decrease in muscle protein breakdown. TE treatment increased expression of AR protein at 1 mo, but expression returned to pre-TE treatment levels by 6 mo. IGF-I protein expression increased at 1 mo and remained increased throughout TE administration. We conclude that physiological and near-physiological increases of testosterone in older men will increase muscle protein anabolism and muscle strength.

aging; muscle strength; lean body mass; insulin-like growth factor I

MOST AGING MEN SHOW A REDUCTION in circulating serum testosterone concentrations (16, 22). This reduction in serum testosterone concentration is a core physiological event in what is termed andropause. Andropause

can be clinically characterized by decreased potency and libido, increased fatigability, and decreased muscle strength (13, 24). A significant decrease in serum total testosterone occurs as early as ages 50–59 (16). This decrease in testosterone production is associated with the loss of lean body mass (LBM) and muscle strength. When men are made hypogonadal with a gonadotropin-releasing hormone analog (14), LBM and muscle strength are lost. Once weakened, older individuals are prone to falls that prevent an independent living status and diminish the quality of life. As the population of older Americans grows, the need to develop therapies to counteract the aging-induced loss in skeletal muscle mass and function becomes critically important.

Previously we demonstrated that testosterone administration primes skeletal muscle for growth by increasing net protein synthesis in the fasted state (10, 18). The logical extrapolation of a continued increase in net protein synthesis is an increase in lean body mass and strength. Bhasin et al. (2) demonstrated that supraphysiological doses of testosterone can induce increases in muscle size and strength in younger men without concomitant exercise. This relationship holds true in relatively hypogonadal populations, where the increase of circulating testosterone increases muscle protein synthesis (23), LBM (3, 20), and muscle strength (3, 23). In an earlier study (23), we demonstrated that 1 mo of testosterone administration increased muscle anabolism and strength in six older men. We also demonstrated that the increase in muscle anabolism was associated with an increase in the expression of intramuscular mRNA for insulin-like growth factor I (IGF-I) (23). Because IGF-I has also been demonstrated to be a potent anabolic hormone (11), the relationship between testosterone administration and IGF-I levels was investigated in the present study.

Previous studies of testosterone administration in older men used a standard clinical dosing paradigm (3, 15, 21). Although this dosing is clinically feasible and

Address for reprint requests and other correspondence: A. A. Ferrando, Depts. of Surgery and Metabolism, Shriners Hospitals for Children, 815 Market St., Galveston, TX 77550 (E-mail: aferrando@utmb.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

convenient, it does not account for individual response to hormone administration. We have previously noted that a given dosage of testosterone administration results in widely varied blood concentrations (23). Although group means often reveal significant increases in testosterone, individual variation may mask a consistency in outcomes. For example, Bhasin et al. (3) and Tenover (21) each used a standard clinical replacement dose in elderly men for up to 3 mo. However, Bhasin et al. demonstrated an increase in muscle strength, whereas Tenover did not. Individual response can be resolved in part by using supraphysiological doses (2); however, these doses may be associated with the potential for increased side effects such as altered lipid profiles (12) or hemodynamic profiles (15). In the present study, we endeavored to adjust individual testosterone concentrations to remain within the mid- to high physiological range. We reasoned that remaining within or near physiological testosterone concentrations would diminish potential side effects while allowing the investigation of testosterone's anabolic effects. We hypothesized that increases in testosterone within or near the physiological range would also stimulate muscle anabolism and increase muscle strength in older men much like previous studies where supplementation resulted in supraphysiological concentrations (2, 15). To accomplish this, we carefully adjusted individual nadir hormone concentrations to remain within the physiological range throughout the 6-mo study. This dosing paradigm permits the investigation of the efficacy of long-term testosterone administration at or near physiological concentrations in older men.

METHODS

Subjects. Twelve healthy, older male subjects were randomly assigned in double-blind fashion to receive either testosterone enanthate (TE) or placebo for 6 mo. Seven subjects [68 ± 3 (SE) yr; 91 ± 5 kg] were randomized to receive TE, whereas five subjects (67 ± 3 yr; 99 ± 7 kg) received a placebo consisting of sesame seed oil. The study was approved by the Institutional Review Board at The University of Texas Medical Branch (UTMB). Informed consent was obtained after the study was explained to each individual. Subjects were selected on the basis of the following inclusion criteria: 1) prostate-specific antigen (PSA) ≤ 4.0 $\mu\text{g/l}$ (6), 2) serum total testosterone ≤ 17 nmol/l (480 ng/dl), 3) serum low-density lipoprotein (LDL) ≤ 200 ng/dl (7), 4) completion of a Bruce treadmill exercise test without significant findings of cardiovascular disease, and 5) no medical illnesses causing disability. The serum testosterone cutoff was chosen because it has been shown that 85% of healthy older men (age 60–98 yr) have serum testosterone concentrations < 17 nmol/l but still in the low-normal range of > 10 nmol/l (1). Exclusion criteria included a history of prostate cancer and severe coronary artery disease (due to the possible hypertrophic and atherogenic effects of testosterone), knee replacement (for reasons of strength determination), or use of a blood anticoagulant, e.g., Coumadin (for fear of excessive bleeding during biopsy and catheterization procedures). Because we wanted to determine the outcomes of testosterone without the confounding effects of exercise (2), we excluded subjects engaged in regular training (defined as 30 min of aerobic or resistance

training activity ≥ 2 days/wk). These exclusion/inclusion criteria were similar to those of previously published studies by our group and others (21, 23).

Experimental protocol. The studies were performed at the General Clinical Research Center (GCRC) at UTMB. Subjects were studied at baseline, after 1 mo, and after 6 mo of treatment. Each GCRC admission consisted of ~ 3 days. On day 1, subjects were admitted in the afternoon and underwent Cybex II isokinetic dynamometer testing for muscular endurance. Subjects followed a standardized protocol that included 15 min of pretest stretching. Muscular endurance was defined as the total work performed for 20 repetitions at $240^\circ/\text{s}$. On the morning of day 2, subjects were weighed in hospital gowns, resting (recumbent) blood pressure was taken, and blood was drawn from the fasted subjects for hematological measures. Subjects were then taken for magnetic resonance imaging (MRI) of the lower body. Leg muscle volume was determined by analysis of images collected by MRI (GE Signa 1.5-Tesla whole body imager; General Electric, Milwaukee, WI) as previously described (9). Image data files generated at the MRI facility were analyzed for appendicular total and muscle volumes using NIH Image software (NIH Image public domain analysis package). Muscle volume (cm^3) was computed as the addition of individual slice areas multiplied by the slice thickness (10 mm). After breakfast, subjects were taken to the UTMB Field House for one-repetition maximum (1RM) determinations for bicep curl, tricep extension, leg extension, and leg curl on specific equipment (Cybex) designed for each movement. Subjects were initially familiarized on the equipment after screening and selection. For 1RM testing, subjects first warmed up on a stationary bike set at 30 W for 10 min. The determination of 1RM was accomplished by increasing the load on each machine until successful completion of the movement was no longer possible. The heaviest load lifted was considered the 1RM. At approximately noon, subjects received dual-energy X-ray absorptiometry (DEXA) to determine LBM and fat mass. Body mass components were determined with regional analysis software as previously described (8). Finally, subjects were referred to the Department of Urology at UTMB for prostate ultrasound and urine flow measurements. Prostate volume was measured by transrectal ultrasound, and urinary flow rate measures were made using a Life-Tech uroflowmeter (Life Tech, Houston, TX).

On day 3, subjects received a stable isotope infusion to determine skeletal muscle protein metabolism. Muscle protein net balance and fractional synthesis rate (FSR) of skeletal muscle were determined by infusion of the stable isotope [^3H]ketoisocaproic acid, arteriovenous sampling, and muscle biopsies as previously described (10). Briefly, skeletal muscle FSR was calculated from the determination of the rate of tracer incorporation into the protein and the enrichment of the intracellular pool as the precursor

$$\text{FSR} = [(E_{p2} - E_{p1}) / (E_M \cdot t)] \cdot 60 \cdot 100$$

where E_{p1} and E_{p2} are the enrichments of the protein-bound [^3H]leucine (from transamination of [^3H]ketoisocaproic acid) from the biopsies at 2 and 5 h of isotope infusion; E_M represents the average intracellular [^3H]leucine enrichment over the time of incorporation; and t is the time in minutes. The factors 60 and 100 are required to express FSR in percent per hour. Each biopsy was divided to be used for both Western blot and isotopic enrichment analyses.

After the isotope infusion study on day 3, subjects were given injections and discharged. Subjects returned every week for fasted blood draw and injections for the first 4 wk and then every 2 wk for the remainder of the study. Serum

total testosterone concentrations were measured on each occasion and adjusted to between 17 and 28 nmol/l (500 and 800 ng/dl; based on the concentration for the visit before each injection) to approximate concentrations found in young men. The aforementioned measurements were made at baseline and at 1 and 6 mo. However, at 1 mo, the MRI, DEXA, and urology measures were omitted. We designed the TE dosing paradigm for weekly injections for the 1st mo so that we could adjust TE doses and establish increased testosterone concentrations by the first measurements that were done at 1 mo. This paradigm was reproduced from our initial study (23). A biweekly injection paradigm would not have allowed TE dose adjustment before the assessments at 1 mo.

Clinical measures. Measurement of clinical parameters (see Table 2) such as testosterone (DPC, Los Angeles, CA), estradiol (DPC), blood lipids (Vitros 250 Chemistry System, Johnson & Johnson, Arlington, TX), PSA, liver function tests (Vitros 250), and hematocrit (Couter Onyx, Beckman Coulter, Brea, CA) were done on a monthly basis by a UTMB clinical laboratory. Subjects were also monitored monthly for breast tenderness and the presence of gynecomastia by history and physical examination. Serum testosterone concentrations were determined by the clinical laboratory, so that adjustments in TE doses could be made on the basis of the previous serum testosterone concentration.

Western blot analysis. Protein was isolated from muscle biopsy samples by slicing frozen muscle in very small pieces with a clean razor blade and thawing the tissue in lysis buffer (150 mM NaCl, 10 mM Tris, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 5 mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 10 μ g/ml aprotinin, 50 μ g/ml leupeptin, 1 μ g/ml pepstatin A) at a concentration of 3 ml of ice cold lysis buffer per gram of tissue. The tissue was homogenized with a Dounce homogenizer (4°C) and centrifuged at 15,000 *g* for 20 min, and the supernatant was removed and centrifuged again to result in total cell lysate. The androgen receptor (AR) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 80 mg of cell lysate run on standard SDS-PAGE gel with a working solution concentration range of 1:15–20. The IGF-I antibody (Santa Cruz Biotechnology) was incubated with 40 mg of cell lysate run on standard SDS-PAGE gel with a working solution concentration of 1:100. The actin “housekeeping” antibody (Sigma) was used with a working solution concentration range of 1:100–200. This anti-actin antibody is a broad-based antibody that recognizes an epitope located on the NH₂-terminal region of actin and demonstrates a broad reactivity among multiple actin isoforms in various species. The housekeeping antibody was used to correct the results for protein loading of the gel. Western analysis allows the direct measurement of protein expression in the muscle biopsy samples.

Statistical analysis. Comparison of 1- and 6-mo measures to baseline values was accomplished by 2-way repeated-measures ANOVA with Dunnett's multiple comparison test. Comparison of clinical outcome values over the 6-mo study period was accomplished by ANOVA with Dunnett's multiple comparison test. Where 1-mo measures were omitted, a paired *t*-test was used to statistically compare 6-mo and baseline values. Statistical significance was $P \leq 0.05$. Data are presented as means \pm SE.

RESULTS

Clinical outcomes. Figure 1 shows the mean testosterone profiles of each group at 2-wk intervals over the 6-mo study period. Table 1 shows the individual tes-

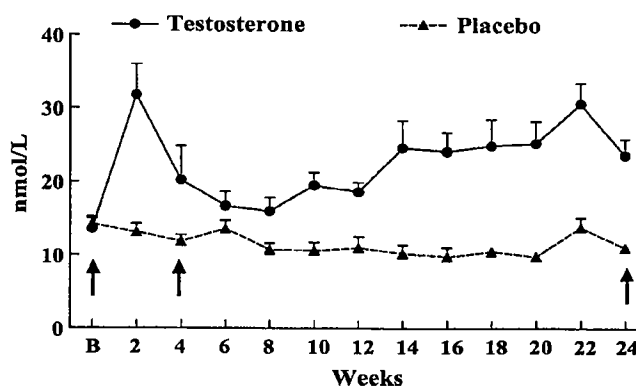


Fig. 1. Serum testosterone profiles throughout treatment. Values are means \pm SE. Testosterone treatment group values were significantly higher at all time points after baseline. Arrows indicate study time points.

tosterone concentrations for each of the seven subjects who received TE and the dose adjustment made for each individual. None were clinically hypogonadal at the beginning of this study. TE injections were adjusted by an independent clinician to maintain levels within the normal range (17–28 nmol/l). As can be seen in Table 1, the serum testosterone concentrations and the doses of TE administered were variable from individual to individual. Following such a paradigm, especially with the use of intramuscular injections, the older men were exposed to serum testosterone concentrations at various times during the 6-mo study that were above the physiological range. Therefore, this study assesses a mix between physiological and near-physiological administration. However, serum testosterone concentrations were greater in the treatment group at all time points after baseline ($P < 0.05$). Serum testosterone did not change in the placebo group. Table 2 delineates subject characteristics and laboratory values over the 6-mo study period. Treatment subjects remained normotensive, and liver function tests, blood lipid profiles, and PSA were unchanged. Estradiol increased upon treatment and, for the most part, remained elevated throughout the 6-mo period without causing breast tenderness or gynecomastia by report or examination. Hematocrit was elevated after 4 mo of TE and remained elevated until the end of the study.

Prostate volume was not significantly increased with TE administration. Prostate volume in the treatment group was 44 ± 15 ml at baseline, whereas the placebo group was 41 ± 8 ml. Six-month values were 47 ± 13 and 35 ± 7 ml, respectively, for the treatment and placebo groups. Urinary flow rate also did not change over time or as a result of treatment. Baseline flow rate was 8.3 ± 1.5 and 8.9 ± 1.3 ml/s, whereas 6-mo values were 7.5 ± 1.4 and 8.7 ± 1.6 ml/s for the treatment and placebo groups, respectively.

Western blot analysis. TE administration significantly increased skeletal muscle AR protein expression at 1 mo ($P < 0.05$), but AR returned to baseline levels at 6 mo. Figure 2 shows a representative autoradio-

Table 1. Serum testosterone concentrations and TE dose adjustments for the 7 older men receiving testosterone

| S | Baseline | 1 wk | 2 wk | 3 wk | 4 wk | 6 wk | 8 wk | 10 wk | 12 wk | 14 wk | 16 wk | 18 wk | 20 wk | 22 wk | 24 wk |
|---|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|-------|
| 1 | 10.7 (100) | 30.9 (100) | 30.9 (100) | 26.3 (100) | 43.3 (150) | 17.6 (125) | 15.8 (200) | 7.4 (200) | 23.7 (200) | 23.9 (200) | 20.6 (200) | 21.0 (250) | 16.4 (250) | 36.9 (300) | 31.8 |
| 2 | 13.9 (100) | 21.8 (100) | 22.1 (100) | 24.1 (50) | 27.0 (150) | 13.3 (150) | 16.5 (150) | 15.4 (200) | 17.6 (200) | 13.9 (250) | 23.2 (250) | 13.9 (300) | 12.2 (350) | 17.5 (400) | 29.3 |
| 3 | 15.9 (100) | 32.3 (100) | 36.8 (100) | 33.6 (50) | 22.4 (150) | 10.7 (150) | 12.6 (200) | 14.7 (200) | 17.6 (250) | 18.4 (250) | 20.1 (300) | 21.9 (300) | 21.8 (350) | 17.1 (350) | 21.2 |
| 4 | 13.3 (100) | 20.5 (100) | 27.8 (150) | 32.8 (150) | 50.3 (150) | 45.5 (150) | 13.9 (100) | 18.2 (200) | 20.6 (200) | 20.7 (200) | 22.1 (250) | 28.6 (250) | 40.2 (250) | 26.6 (200) | 26.4 |
| 5 | 11.4 (100) | 24.4 (100) | 22.2 (100) | 19.6 (100) | 24.0 (200) | 9.4 (200) | 12.0 (250) | 14.8 (300) | 21.0 (350) | 18.1 (350) | 22.0 (400) | 30.3 (400) | 25.6 (400) | 26.8 (400) | 37.3 |
| 6 | 9.6 (100) | 13.8 (100) | 18.7 (150) | 26.6 (150) | 22.3 (200) | 23.8 (200) | 19.4 (200) | 18.9 (250) | 11.4 (250) | 14.9 (300) | 17.9 (300) | 21.5 (400) | 24.5 (400) | 18.2 (400) | 25.4 |
| 7 | 13.2 (100) | 16.8 (100) | 22.1 (150) | 29.8 (150) | 33.0 (200) | 20.2 (200) | 26.6 (250) | 22.6 (250) | 25.0 (300) | 20.6 (300) | 46.3 (300) | 32.7 (300) | 33.6 (300) | 32.9 (300) | 42.7 |

Nos. are testosterone concentrations in nmol/l; nos. in parentheses are doses of testosterone enanthate (TE) administered (mg) at that visit. S, subject. Testosterone dose adjustments were made on the basis of the preceding testosterone concentrations; i.e., an adjustment at 8 wk was based on the 6-wk testosterone concentration.

gram of a Western blot for skeletal muscle AR from a subject receiving testosterone and a graph of the densitometry data from the treatment group. There was no correlation between the serum testosterone concentration at 1 mo and the change of AR expression from baseline to 1 mo for individuals. IGF-I protein expression in skeletal muscle increased at 1 mo and remained elevated at 6 mo ($P < 0.05$; Fig. 3). AR and IGF-I protein expression did not change in the placebo group (data not shown).

Physiological outcomes. The net balance of muscle protein was less negative in the fasted state in the treatment group throughout TE administration (Fig. 4; $P < 0.05$), but still less than zero. In other words,

treatment subjects were less catabolic when fasting than those in the placebo group. The more favorable net balance was due to a decrease in fasting protein breakdown, as fractional synthetic rate of muscle protein remained constant throughout (0.071 ± 0.02 to 0.084 ± 0.013 to $0.062 \pm 0.016\%/h$ at baseline and 1 and 6 mo, respectively).

The resultant improvement in net protein balance led to an increase in LBM. Table 3 outlines the changes in LBM and muscle strength over the 6-mo study period. The treatment group demonstrated increases in total and leg LBM, whereas the percentage of total body fat diminished. Leg muscle volume by MRI was also increased significantly after 6 mo of TE adminis-

Table 2. Subject characteristics and laboratory values during 6 mo of testosterone or placebo treatment

| | Baseline | 1 mo | 2 mo | 3 mo | 4 mo | 5 mo | 6 mo |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Weight, kg | 91 \pm 5 | 92 \pm 5 | 92 \pm 5 | 92 \pm 5 | 92 \pm 5 | 92 \pm 5 | 91 \pm 6 |
| Systolic BP, mmHg | 99 \pm 7 | 98 \pm 6 | 98 \pm 7 | 98 \pm 7 | 96 \pm 6 | 96 \pm 6 | 96 \pm 6 |
| Diastolic BP, mmHg | 141 \pm 7 | 144 \pm 4 | 154 \pm 5 | 150 \pm 4 | 156 \pm 5 | 152 \pm 6 | 149 \pm 3 |
| ALT U/l (9–51) | 140 \pm 8 | 148 \pm 5 | 155 \pm 3 | 150 \pm 7 | 154 \pm 4 | 147 \pm 8 | 148 \pm 5 |
| AST U/l (13–40) | 78 \pm 3 | 79 \pm 3 | 82 \pm 4 | 85 \pm 4 | 87 \pm 3 | 84 \pm 3 | 85 \pm 3 |
| Chol. (mmol/l) (3.1–5.2) | 82 \pm 6 | 81 \pm 7 | 79 \pm 5 | 83 \pm 5 | 83 \pm 6 | 78 \pm 5 | 79 \pm 4 |
| HDL (mmol/l) (0.78–1.8) | 32 \pm 3 | 30 \pm 2 | 27 \pm 2 | 27 \pm 3 | 29 \pm 3 | 32 \pm 3 | 28 \pm 3 |
| LDL (mmol/l) (2.1–5.69) | 36 \pm 10 | 37 \pm 9 | 35 \pm 6 | 34 \pm 6 | 33 \pm 6 | 29 \pm 2 | 30 \pm 4 |
| PSA, μ g/l (<4) | 23 \pm 2 | 23 \pm 1 | 24 \pm 3 | 22 \pm 2 | 24 \pm 2 | 25 \pm 2 | 24 \pm 2 |
| E ₂ , pmol/l (48–173) | 24 \pm 7 | 27 \pm 7 | 25 \pm 6 | 25 \pm 5 | 24 \pm 5 | 19 \pm 5 | 22 \pm 5 |
| Hct, % (37–50) | 4.81 \pm 0.62 | 4.81 \pm 0.28 | 4.71 \pm 0.21 | 4.65 \pm 0.28 | 4.73 \pm 0.16 | 4.84 \pm 0.16 | 4.50 \pm 0.23 |
| | 5.15 \pm 0.34 | 5.17 \pm 0.44 | 5.30 \pm 0.31 | 5.3 \pm 0.26 | 4.84 \pm 0.28 | 5.02 \pm 0.26 | 5.12 \pm 0.26 |
| | 1.14 \pm 0.13 | 1.01 \pm 0.10 | 1.11 \pm 0.13 | 1.06 \pm 0.10 | 1.06 \pm 0.13 | 1.09 \pm 0.13 | 0.96 \pm 0.13 |
| | 0.88 \pm 0.08 | 0.96 \pm 0.08 | 0.91 \pm 0.05 | 0.88 \pm 0.05 | 0.85 \pm 0.08 | 0.93 \pm 0.08 | 0.88 \pm 0.08 |
| | 2.77 \pm 0.34 | 2.79 \pm 0.28 | 2.79 \pm 0.18 | 2.71 \pm 0.26 | 2.77 \pm 0.6 | 2.92 \pm 0.18 | 2.69 \pm 0.23 |
| | 2.97 \pm 0.54 | 3.23 \pm 0.47 | 3.36 \pm 0.39 | 2.92 \pm 0.57 | 2.82 \pm 0.47 | 3.02 \pm 0.34 | 3.05 \pm 0.40 |
| | 1.4 \pm 0.4 | 1.9 \pm 0.4 | 2.1 \pm 0.7 | 2.0 \pm 0.7 | 2.1 \pm 0.4 | 2.0 \pm 0.5 | 2.3 \pm 0.9 |
| | 1.2 \pm 0.4 | 1.4 \pm 0.4 | 1.2 \pm 0.4 | 1.0 \pm 0.4 | 1.1 \pm 0.4 | 1.2 \pm 0.3 | 1.3 \pm 0.4 |
| | 103 \pm 7 | 272 \pm 33* | 114 \pm 1 | 154 \pm 26* | 187 \pm 26* | 169 \pm 18* | 242 \pm 40* |
| | 110 \pm 15 | 14 \pm 7 | 92 \pm 15 | 103 \pm 18 | 92 \pm 11 | 103 \pm 11 | 117 \pm 15 |
| | 40 \pm 0.8 | 40 \pm 0.7 | 43 \pm 0.8 | 44 \pm 0.9 | 45 \pm 0.1* | 46 \pm 0.8* | 44 \pm 1* |
| | 40 \pm 0.7 | 41 \pm 0.3 | 41 \pm 0.6 | 43 \pm 0.7 | 42 \pm 1 | 41 \pm 0.4 | 40 \pm 0.8 |

Values are means \pm SE. For each test parameter, the first line represents values for the testosterone group ($n = 7$), and the second line represents values for the placebo group ($n = 5$). BP, blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Chol., total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PSA, prostate-specific antigen; E₂, estradiol; Hct, hematocrit. Normal ranges are given in parentheses by the tests. *Statistical significance from the placebo group at each time point as determined by ANOVA with Dunnett's multiple comparison test.

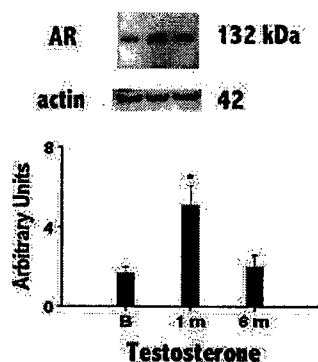


Fig. 2. Androgen receptor (AR) protein expression in skeletal muscle during 6 mo of testosterone administration in older men. *Top*: representative Western blot from one of the 7 subjects assessed for protein expression of AR by use of standard Western analysis. Actin was used as an internal control for protein loading. *Bottom*: means \pm SE from the 7 subjects that received testosterone. Five subjects who received placebo demonstrated no change throughout the study in AR expression (data not shown). Data are expressed as arbitrary units calculated as the ratio of the band densities of AR over the band densities of actin. *Statistical significance was determined by ANOVA, $P \leq 0.05$.

tration. All 1RM strength scores increased in the treatment group after 6 mo of TE. Muscular endurance, as tested by an isokinetic dynamometer, did not increase at 1 or 6 mo.

DISCUSSION

This study demonstrates that testosterone increases within or near the physiological range can produce increases in muscle anabolism, LBM, and muscle strength similar to supraphysiological administration. We monitored serum testosterone concentrations and adjusted the dose of TE to maintain testosterone concentrations in older men in ranges comparable with those of younger men. During the 6 mo of TE administration, some subjects experienced testosterone concentrations that exceeded the physiological; however,

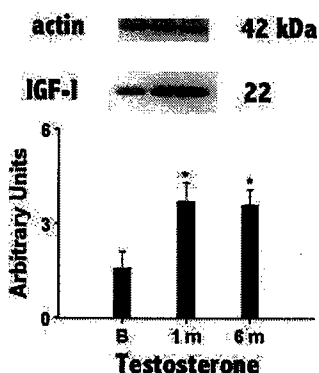


Fig. 3. Insulin-like growth factor I (IGF-I) protein expression in skeletal muscle during 6 mo of testosterone administration in older men. *Top*: representative Western blot from one of 7 subjects assessed for expression of IGF-I by use of standard Western analysis. *Bottom*: mean data from the 7 subjects receiving testosterone administration. Five subjects who received placebo demonstrated no change throughout the study in IGF-I expression (data not shown). Data were derived as described in Fig. 2.

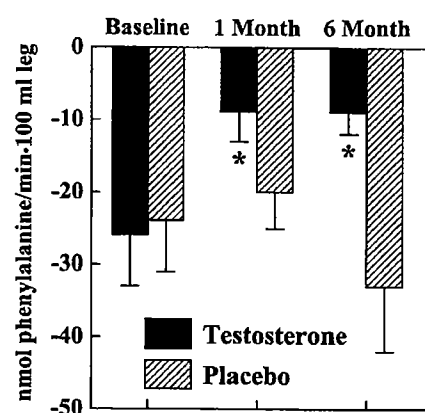


Fig. 4. Fasting net phenylalanine balance across the leg. Phenylalanine net balance describes the net balance between muscle protein synthesis and breakdown. *Significantly less negative than the placebo group and baseline testosterone, by ANOVA, $P < 0.05$.

testosterone concentrations were consistently maintained above baseline values. The older men in this study demonstrated an increase in LBM that was comparable to that achieved with a standard replacement regimen that resulted in higher testosterone concentrations (5). We also found that, similar to younger men (2), testosterone will increase muscle anabolism and strength in older men. The strength increases of the older men in this study were greater than those demonstrated with standard replacement paradigms (15, 21) or with testosterone patch administration over

Table 3. Absolute changes in body mass and muscle strength with 6 mo of testosterone treatment

| Variable, units | Treatment | | P Value |
|--|--------------------------|-------------------------------|----------|
| | Testosterone | Placebo | |
| ¹ Total LBM, kg | $\uparrow 4.2 \pm 0.6$ | $\downarrow 2.0 \pm 1.0$ | <0.001 |
| ¹ Leg LBM, kg | $\uparrow 1.6 \pm 0.4$ | $\downarrow 1.3 \pm 0.6$ | 0.003 |
| ¹ Arm LBM, kg | $\uparrow 1.6 \pm 0.6$ | $\downarrow 1.4 \pm 1.4$ | 0.056 |
| ¹ Body fat, % | $\downarrow 3.6 \pm 0.7$ | $\uparrow 0.3 \pm 1.7$ | 0.039 |
| ² Leg muscle volume, ml | $\uparrow 488 \pm 76$ | $\downarrow 96 \pm 155$ | 0.04 |
| ³ Bicep curl, kg | | | |
| 1 mo | $\uparrow 3.6 \pm 2.3$ | $\downarrow 0.5 \pm 2.0$ | 0.16 |
| 6 mo | $\uparrow 9.1 \pm 1.5$ | $\downarrow 0.9 \pm 1.5$ | 0.002 |
| ³ Tricep extension, kg | | | |
| 1 mo | $\uparrow 4.9 \pm 1.7$ | $\uparrow 2.3 \pm 1.6$ | 0.33 |
| 6 mo | $\uparrow 10.4 \pm 2.1$ | $\downarrow 0.9 \pm 1.2$ | <0.001 |
| ³ Leg curl, kg | | | |
| 1 mo | $\uparrow 5.5 \pm 2.4$ | $\uparrow 1.8 \pm 1.3$ | 0.11 |
| 6 mo | $\uparrow 7.5 \pm 2.1$ | $\leftrightarrow 0.0 \pm 4.0$ | 0.051 |
| ³ Leg extension, kg | | | |
| 1 mo | $\uparrow 6.5 \pm 3.1$ | $\uparrow 4.5 \pm 1.4$ | 0.73 |
| 6 mo | $\uparrow 15.3 \pm 5.2$ | $\leftrightarrow 0.0 \pm 3.1$ | 0.015 |
| Isokinetic endurance knee extension, dominant leg, J | | | |
| 1 mo | $\uparrow 2.3 \pm 3.1$ | $\uparrow 3.4 \pm 6.8$ | .313 |
| 6 mo | $\uparrow 17.6 \pm 9.8$ | $\uparrow 6.0 \pm 7.5$ | .344 |

Values are means \pm SE. LBM, lean body mass. ¹Dual-energy X-ray absorptiometry; ²magnetic resonance imaging; ³one-repetition maximum on Cybex equipment with both limbs. P value denotes differences between treatments; \uparrow or \downarrow indicates direction of numerical change.

36 mo (20). Our data suggest that a standard paradigm of testosterone administration that does not include individual dose adjustment may not always achieve desired outcomes if the subjects have not received adequate testosterone to stimulate metabolic changes in muscle. Because we studied only a small number of subjects, we cannot draw any conclusions regarding the risk-to-benefit ratio of testosterone administration in older men. However, we found no significant side effects in our small group other than an increase in hematocrit. Our data indicate that testosterone can improve muscle strength in older men when careful dosing ensures sustained blood testosterone increases. Our first study demonstrated that short-term administration with standard replacement dosages resulted in LBM and strength increases (23). The present study indicates that these LBM and strength increases can be maintained over 6 mo with careful dose adjustments that ensure primarily physiological testosterone levels. This study also demonstrates that the muscle's response to testosterone changes over the 6-mo period of administration, indicating that alternative paradigms of testosterone administration (i.e., cyclic administration) can be of physiological benefit.

Testosterone administration resulted in some noteworthy effects on AR and IGF-I expression in skeletal muscle. AR protein expression was increased after 1 mo of TE but had returned to pretreatment levels by 6 mo. Physiologically, it is logical that androgen would enhance its own receptor expression as it stimulates muscle metabolism. We previously noted an upregulation of AR expression with oxandrolone administration (18) in young males, which also occurred concomitantly with an increase in muscle protein synthesis. The return of AR expression to pretreatment values after 6 mo of continuous androgen administration indicates a steady-state adaptation to the treatment paradigm. There is also the possibility that the AR response is nothing more than a response to the dosing paradigm. At 1 mo, older subjects were receiving TE weekly rather than every 2 wk, and their mean serum testosterone concentrations were more in the supraphysiological range than they were at 6 mo. However, this relationship is weakened by the fact that individual testosterone concentrations at 1 mo did not correlate with the change in AR expression from baseline to 1 mo. This pattern of AR expression raises the possibility that cycling of testosterone administration could produce effects on skeletal muscle analogous to continuous administration. Such a paradigm would be beneficial by administering significantly less testosterone for similar anabolic outcomes, thus minimizing the possibility of side effects.

IGF-I accompanies increases in muscle mass and strength (17). In frail elderly, progressive resistance training that increases muscle mass and strength also increases intramuscular IGF-I concentrations (19). Clinically, we previously demonstrated that older men given testosterone for 1 mo increased IGF-I transcripts in muscle while decreasing the inhibitory IGF-binding protein (23). The present study agrees with our previ-

ous work in that IGF-I protein expression increased at 1 mo and further demonstrates that this increase was maintained throughout the 6 mo of testosterone administration. This confirms that the increase in IGF-I mRNA noted in our earlier study (23) translates into an actual increase of IGF-I protein. A corollary to these studies found that young men who were made hypogonadal for 10 wk by Lupron showed a decrease in muscle strength and a decrease in intramuscular IGF-I mRNA concentration (14). Taken together, these data indicate a mechanistic importance of IGF-I on muscle anabolism.

Although the intracellular mechanism stimulating muscle protein anabolism requires further clarification, it is clear that testosterone improves net protein balance of skeletal muscle. This effect is pronounced in the fasted state as net protein balance becomes less negative. We have previously demonstrated (10, 18) that one of the primary effects of testosterone (during fasting) is the efficient reutilization of intracellular amino acids (derived from protein breakdown) for protein synthesis. However, the present study demonstrates that, even if breakdown is decreased, ample amino acid precursors are present to support the initial rate of protein synthesis. Thus testosterone administration may ameliorate the loss of skeletal muscle nitrogen during fasting in this older population by preventing the loss of intracellular amino acids. Not only is the appearance of amino acids from protein breakdown reduced, but those that are derived from protein breakdown are efficiently utilized to maintain protein synthesis, as we have previously demonstrated (10, 18). This retention of nitrogen during fasting, when combined with the anabolic stimulus of a meal alone (4, 25), may lead to muscle (LBM) accretion over time and explain the anabolic effects of chronic testosterone administration.

In summary, the present study demonstrates that careful and near-physiological testosterone administration in older men will increase LBM and muscle strength similarly to younger men. However, further consideration should be given to the specific androgen and length and type of administration regimen to be used in older men and to large-scale studies initiated to determine the risk-to-benefit ratio of testosterone administration in older men.

This study was supported by National Institutes of Health Grants AG/AR-11000 (R. J. Urban), M01-RR-00073 (General Clinical Research Center, University of Texas Medical Branch), GM-57295 (A. A. Ferrando), and Shriners Hospitals for Children Grant 8940 (R. R. Wolfe).

REFERENCES

1. Abbasi AA, Drinka PJ, Mattson DE, and Rudman D. Low circulating levels of insulin-like growth factors and testosterone in chronically institutionalized elderly men. *J Am Geriatr Soc* 41: 975-982, 1993.
2. Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Tricker R, Shirazi A, and Casaburi R. The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med* 335: 1-7, 1996.

3. Bhasin S, Storer TW, Berman N, Yarasheski KE, Clevenger B, Phillips J, Lee WP, Bunnell TJ, and Casaburi R. Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. *J Clin Endocrinol Metab* 82: 407–413, 1997.
4. Biolo G, Tessari P, Inchiostro S, Bruttomesso D, Fongher C, Sabadin L, Fratton MG, Valerio A, and Tiengo A. Leucine and phenylalanine kinetics during mixed-meal ingestion: a multiple tracer approach. *Am J Physiol Endocrinol Metab* 262: E455–E463, 1992.
5. Brodsky IG, Balagopal P, and Nair KS. Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men—a clinical research center study. *J Clin Endocrinol Metab* 81: 3469–3475, 1996.
6. Catalona WJ, Smith DS, Ratliff TL, Dodds KM, Coplen DE, Yuan JJ, Petros JA, and Andriole GL. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer [published erratum appears in *N Engl J Med* 1991 Oct 31, 325: 1324]. *N Engl J Med* 324: 1156–1161, 1991.
7. Denke MA and Grundy SM. Hypercholesterolemia in elderly persons: resolving the treatment dilemma. *Ann Intern Med* 112: 780–792, 1990.
8. Ferrando AA, Lane HW, Stuart CA, Davis-Street J, and Wolfe RR. Prolonged bed rest decreases skeletal muscle and whole body protein synthesis. *Am J Physiol Endocrinol Metab* 270: E627–E633, 1996.
9. Ferrando AA, Stuart CA, Brunder DG, and Hillman GR. Magnetic resonance imaging quantitation of changes in muscle volume during 7 days of strict bed rest. *Aviat Space Environ Med* 66: 976–981, 1995.
10. Ferrando AA, Tipton KD, Doyle D, Phillips SM, Cortiella J, and Wolfe RR. Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. *Am J Physiol Endocrinol Metab* 275: E864–E871, 1998.
11. Fryburg DA, Jahn LA, Hill SA, Oliveras DM, and Barrett EJ. Insulin and insulin-like growth factor-I enhance human skeletal muscle protein anabolism during hyperaminoacidemia by different mechanisms. *J Clin Invest* 96: 1722–1729, 1995.
12. Haupt HA and Rovere GD. Anabolic steroids: a review of the literature. *Am J Sports Med* 12: 469–484, 1984.
13. Mastrogiacomo I, Feghali G, Foresta C, and Ruzza G. Andropause: incidence and pathogenesis. *Arch Androl* 9: 293–296, 1982.
14. Mauras N, Hayes V, Welch S, Rini A, Helgeson K, Dokler M, Veldhuis JD, and Urban RJ. Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. *J Clin Endocrinol Metab* 83: 1886–1892, 1998.
15. Morley JE, Perry HM III, Kaiser FE, Kraenzle D, Jensen J, Houston K, Mattammal M, and Perry HM Jr. Effects of testosterone replacement therapy in old hypogonadal males: a preliminary study. *J Am Geriatr Soc* 41: 149–152, 1993.
16. Moroz EV and Verkhatsky NS. Hypophyseal-gonadal system during male aging. *Arch Gerontol Geriatr* 4: 13–19, 1985.
17. Sheffield-Moore M. Androgens and the control of skeletal muscle protein synthesis. *Ann Med* 32: 181–186, 2000.
18. Sheffield-Moore M, Urban RJ, Wolf SE, Jiang J, Catlin DH, Herndon DN, Wolfe RR, and Ferrando AA. Short-term oxandrolone administration stimulates net muscle protein synthesis in young men. *J Clin Endocrinol Metab* 84: 2705–2711, 1999.
19. Singh MA, Ding W, Manfredi TJ, Solares GS, O'Neill EF, Clements KM, Ryan ND, Kehayias JJ, Fielding RA, and Evans WJ. Insulin-like growth factor I in skeletal muscle after weight-lifting exercise in frail elders. *Am J Physiol Endocrinol Metab* 277: E135–E143, 1999.
20. Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Lenrow DA, Holmes JH, Dlewati A, Santanna J, Rosen CJ, and Strom BL. Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. *J Clin Endocrinol Metab* 84: 2647–2653, 1999.
21. Tenover JS. Effects of testosterone supplementation in the aging male. *J Clin Endocrinol Metab* 75: 1092–1098, 1992.
22. Tenover JS, Matsumoto AM, Plymate SR, and Bremner WJ. The effects of aging in normal men on bioavailable testosterone and luteinizing hormone secretion: response to clomiphene citrate. *J Clin Endocrinol Metab* 65: 1118–1126, 1987.
23. Urban RJ, Bodenbun YH, Gilkison C, Foxworth J, Coggan AR, Wolfe RR, and Ferrando A. Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. *Am J Physiol Endocrinol Metab* 269: E820–E826, 1995.
24. Urban RJ and Veldhuis VJ. Hypothalamo-pituitary concomitants of aging. In: *The Endocrinology of Aging*, edited by Sowers JR and Felicetta JV. New York: Raven, 1988, p. 44–74.
25. Volpi E, Mittendorfer B, Wolf SE, and Wolfe RR. Oral amino acids stimulate muscle protein anabolism in the elderly despite higher first-pass splanchnic extraction. *Am J Physiol Endocrinol Metab* 277: E513–E520, 1999.

Oral Testosterone in Oil Plus Dutasteride in Men: A Pharmacokinetic Study

John K. Amory and William J. Bremner

Center for Research in Reproduction and Contraception, Divisions of General Internal Medicine and Endocrinology, Metabolism, and Nutrition, University of Washington Medical School, Seattle, Washington 98195

Testosterone (T) is not administered orally, because it has been reported to be rapidly metabolized by the liver. We hypothesized that sufficient doses of T or T enanthate (TE), administered orally in oil, would result in clinically useful elevations in serum T. We also hypothesized that coadministration of dutasteride (D) with T or TE would minimize increases in serum DHT seen previously with oral administration. Therefore, we conducted a pharmacokinetic study of oral T and TE in oil, with and without concomitant D, in normal men whose T production had been temporarily suppressed by the GnRH antagonist acyline. Thirteen healthy men (mean age, 24 ± 6 yr) were enrolled and assigned to oral T ($n = 7$) and oral TE ($n = 6$) groups and were administered 200, 400, or 800 mg of either T or TE in sesame oil in the morning on 3 successive days 24 h after receiving acyline. Blood samples for measurement of serum T and dihydrotestos-

terone were obtained before T or TE administration and 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h after administration. Subjects were then administered D for 4 d before repeating the sequence of T or TE doses with D. Serum T was significantly increased in a dose-dependent fashion with the administration of oral T or TE in oil. Coadministration of D with oral T or TE significantly increased the 24-hr average serum T levels compared with administration of T or TE alone [average serum T after 400 mg dose, 8.7 ± 3.0 nmol/l (T) and 8.3 ± 5.7 nmol/l (TE) vs. 16.1 ± 5.8 nmol/l (T + D) and 15.0 ± 8.8 nmol/l (TE + D); $P < 0.05$ for T vs. T and D]. The administration of oral T or TE in oil combined with D results in unexpected and potentially therapeutic increases in serum T. Additional studies of this combination as a novel form of oral androgen therapy are warranted. (*J Clin Endocrinol Metab* 90: 2610–2617, 2005)

TESTOSTERONE (T) IS crucial for male health. The normal male testes produce 4–8 mg T daily (1, 2). Depending on age, 2.5–10% of men have T levels below the normal range (3). T has effects on a variety of tissues, including brain, liver, muscle, bone and bone marrow, blood vessels, skin, prostate, and penis. Men with T deficiency have symptoms of depression, reduced libido, and low energy and suffer from anemia, osteoporosis, and debilitating muscle weakness. These men require T replacement therapy to improve well-being, maintain bone and muscle mass, and retain healthy sexual function (4–8), yet there is no acceptable form of oral T for therapy in the United States.

Most T regimens in the United States depend on parenteral injections, skin patches, gels, or buccal tablets (9–11), because currently available oral forms of T are alkylated and cause liver toxicity when used long term (11–16). Injections are administered im every 1–3 wk and can be painful (17). Some T patches can cause moderate to severe skin reactions due to the vehicle that facilitates T absorption across the skin (18). The T gels are effective and generally well accepted by patients, but are expensive, and care must be taken to avoid inadvertent exposure to women and children (19).

Oral administration of unmodified T at doses up to 100 mg have little effect on serum T levels in T-deficient men (20, 21); however, 200-mg doses of oral T have been shown to elevate

serum T levels to the low normal range for up to 8 h (22, 23). At the time, these serum T levels were thought to be insufficient for clinical use, and research into using unmodified oral T was largely abandoned.

Testosterone undecanoate (TU) is a T ester currently given orally in oil and used clinically in Europe and Canada for the treatment of T deficiency. When administered orally, TU therapy results in therapeutic increases in serum T; however, it also results in elevations in serum dihydrotestosterone (DHT) well above the normal range (24–27). Because DHT is required for cell growth within the prostate, concern has been raised about the potential for long-term harm associated with oral TU therapy from the elevated levels of serum DHT; however, no increased risk of prostate disease has been reported to date.

Because the androgen TU is absorbed well in oil, we believed that other androgens such as T enanthate (TE) and potentially T itself might be well absorbed if also administered orally in oil. Moreover, because the recently available 5α -reductase inhibitor, dutasteride (D), lowers serum DHT levels more than 90% by inhibiting both isozymes of 5α -reductase (28), we hypothesized that oral administration of the combination of higher doses of unmodified T or the T ester, TE, in oil when combined with D would be safe and result in therapeutic serum T levels. In addition, we hypothesized that the concomitant administration of the 5α -reductase inhibitor D with T or TE would further increase serum T levels while minimizing the elevations in serum DHT seen after oral administration of oral androgens such as TU. If effective, we believed that this novel means of T therapy would allow for selective androgen therapy in men with T

First Published Online February 15, 2005

Abbreviations: D, Dutasteride; DHT, dihydrotestosterone; E2, estradiol; T, testosterone; $t_{1/2}$, half-life; TE, testosterone enanthate; TU, testosterone undecanoate.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

deficiency. Therefore, we conducted a pilot study of the oral administration of single doses of T and TE with and without concomitant administration of D to determine the pharmacokinetics and safety of single high doses oral T in oil in healthy men rendered temporarily hypogonadal with the GnRH antagonist acyline.

Materials and Methods

Subjects

Fourteen healthy, normal male volunteers between 18 and 45 yr of age were recruited through local news media (newspaper and radio) and college campus bulletin boards and enrolled in the study. The inclusion criteria were no prior medical illnesses, normal physical examination, and routine hematology, blood chemistry, and liver function. Exclusion criteria included regular use of any medication; abnormal serum T, DHT, or estradiol (E2); or previous or current ethanol, illicit drug, or anabolic steroid abuse. A total of 16 men were evaluated for eligibility. Of these, 14 men were potentially eligible and agreed to participate in the study. The two men who did not enroll in the study were excluded for elevated bilirubin (one subject) and use of finasteride (for the treatment of male-pattern baldness). One enrolled subject failed to appear for his acyline injection and was therefore not studied further; thus, 13 men completed the study period. The institutional review board of University of Washington approved all study procedures, and subjects gave written informed consent before screening.

Study design

Participants were randomly assigned to one of two groups: 1) oral T in sesame oil, or 2) oral TE in sesame oil (Delatestryl, BTG Pharmaceuticals, Iselin, NJ) at a concentration of 200 mg/ml. A sample size of seven subjects per group was estimated to have an 80% power with an α of 0.05 to detect a 50% in the change in serum T area under the curve between a given dose of T and T plus D or between TE and TE plus D. The oral T in sesame oil was manufactured by the compounding pharmacy at University of Washington. Briefly, micronized T (U.S.P. grade, Spectrum Quality Projects, Gardena, CA) was suspended at 100 mg/ml in sesame oil (N.F. grade, Spectrum Quality Projects) and mixed thoroughly on a magnetic stir plate to create a homogenous T/sesame oil emulsion. The compounding pharmacist then drew up the emulsion into syringes at the desired dose levels (200, 400, and 800 mg) immediately before treatment. The syringe was sent to the Clinical Research Unit, where it was vigorously mixed (by shaking) with milk and administered to the subject. The dose of oral TE in sesame oil was normalized for the T content, so that the subjects in the TE group (molecular weight, 397) were administered 276, 554, and 1108 mg TE, corresponding to 200, 400, and 800 mg T.

The drug exposure period lasted 11 d (Fig. 1). On d 0, subjects received a single injection of the GnRH antagonist acyline (300 μ g/kg, sc), which has been shown to suppress T production in normal men for a minimum of 15 d (29). One, 2, and 3 d after acyline administration, subjects drank 200, 400, or 800 mg T or 276, 554, or 1108 mg TE. Subjects self-administered D (0.5 mg, orally, once daily) on d 5–10 after acyline injection, and doses of T and TE were repeated on days 8, 9, and 10. For safety, subjects

underwent daily testing of liver function (aspartate aminotransferase, bilirubin, and alkaline phosphatase), kidney function (urea nitrogen and creatinine), and hemopoiesis (hemoglobin and hematocrit).

Measurements

After treatment on d 1, 2, 3, 8, 9, and 10, subjects had blood drawn via a heparin-locked iv line at 30 min and 1, 2, 4, 6, 8, 10, 12, and 24 h for measurement of serum T, DHT, E2, and SHBG. Total T was measured by a RIA (Diagnostic Products Corp., Webster, TX). The assay had a sensitivity of 0.35 nmol/liter; interassay variations for low, medium, and high pools of 13.6%, 6.1%, and 6.8%, respectively; and intraassay variations of 10.0%, 5.3%, and 6.6%. The normal range was 8.7–33 nmol/liter. DHT was measured using an RIA kit (Diagnostic Systems Laboratory, Inc., Los Angeles, CA). The sensitivity of this assay was 0.043 nmol/liter, and the intraassay variations for medium and low range pools were 9.9% and 11%, respectively, with interassay coefficients of variations of 19% and 25%. The normal range for serum DHT was 1.0–2.9 nmol/liter. SHBG was measured by RIA (Delphia, Wallac Oy, Turku, Finland). The sensitivity of this assay was 0.2 nmol/liter, and the interassay variations for low, medium, and high pools were 31%, 10.6%, and 6.8%, respectively; the intraassay variations were 3.8%, 1.7%, and 2.2%. The normal range was 3.2–47 nmol/liter. The normal ranges for T, DHT, and SHBG were determined in our laboratory using serum samples obtained from 100 normal men, aged 20–50 yr. Serum E2 was measured in the laboratory of Dr. David Hess (Oregon National Primate Research Center, Portland, OR) with an Elecsys 2010 Platform (Roche, Indianapolis, IN). The sensitivity of this assay was 5.5 pmol/liter, intraassay variations were 3.7%, and 2.8% for medium and high range values, and the interassay coefficient of variation was 4.7%. The normal range for serum E2 in this assay in men was 40–220 pmol/liter.

Statistics

Serum hormone levels at each time point for each dose of T or TE with or without D were compared using a Wilcoxon sign-rank test. Pharmacokinetic parameters between successive doses of T or TE with or without D were compared using a Wilcoxon sign-rank test with a Bonferroni correction for repeated measures (effective α = 0.01). The average concentration during the 24-h period after treatment, the maximum concentration after dosing, time to maximum concentration, area under the curve, and elimination phase half-life ($t_{1/2}$) were calculated using a pharmacokinetics program (PK Solutions, Golden, CO). Statistical analyses were performed using STATA (College Park, TX).

Results

Subjects

Fourteen men were enrolled in the study; seven were randomized to the T group, and seven were randomized to the TE group, but one man assigned to the TE group failed to report for his acyline injection. Therefore, seven men completed the T arm, and six completed the TE arm of the study (Table 1). Except for the subject who failed to appear for his acyline injection, all subjects completed the drug exposure

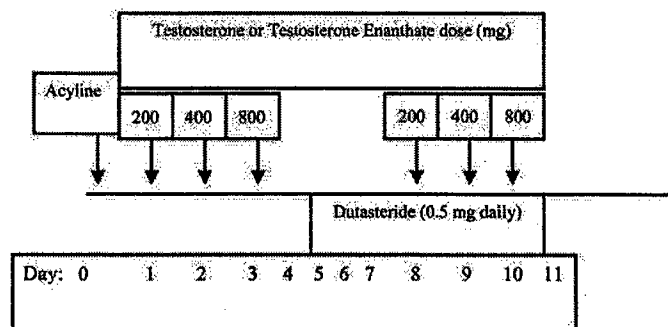


FIG. 1. Study design.

TABLE 1. Baseline characteristics of study subjects by group

| | T group (n = 7) | TE group (n = 6) |
|--------------------------|-----------------|------------------|
| Age (yr) | 24.2 ± 8.7 | 24.7 ± 6.7 |
| Weight (kg) | 77 ± 4.0 | 89 ± 16 |
| Height (cm) | 182 ± 9 | 186 ± 11 |
| BMI (kg/m ²) | 23.3 ± 2.3 | 25.8 ± 4.2 |
| Total T (nmol/liter) | 22.7 ± 8.0 | 17.0 ± 5.8 |
| DHT (nmol/liter) | 1.24 ± 0.46 | 1.1 ± 0.5 |
| SHBG (nmol/liter) | 33.2 ± 9.84 | 24.0 ± 10.7 |
| Free T (pmol/liter) | 435 ± 156 | 341 ± 92 |
| E2 (pmol/liter) | 132 ± 17 | 121 ± 31 |

Values are the mean ± SD. BMI, Body mass index (weight in kilograms/[height in meters]²).

period. There were no serious adverse effects during the study. Nine of the subjects experienced transient mild pruritis at the site of the acyline injection, which resolved in all cases within 1 h of the injection. Eight subjects complained of mild, transient hot flash symptoms toward the end of the study period, presumably due to low T levels; however, no subject complained of feelings of anger, aggression, or irritability during treatment. There were no adverse gastrointestinal symptoms associated with oral T or oral TE in oil. One subject developed a small area of gynecomastia ($<1 \times 1$ cm) immediately under the nipple during the treatment period, but this resolved during follow-up. There were no changes in serum markers of liver or kidney function or in

the hematocrit or hemoglobin during the treatment phase or at follow-up. Furthermore, no significant changes in blood pressure or pulse were observed. T and gonadotropin levels returned to baseline in all subjects during the follow-up period (data not shown). No subjects were lost to follow-up.

Serum T

All subjects were suppressed to castrate levels of T by 24 h after acyline administration (d 0 T, 20.0 ± 7.4 ; d 1 T, 2.3 ± 0.5 nmol/liter; $P < 0.0001$). There was no difference in serum T levels 24 h after acyline between groups [2.3 ± 0.7 (T) vs. 2.3 ± 0.8 (TE); $P = 0.9$]. In addition, mean serum T levels before

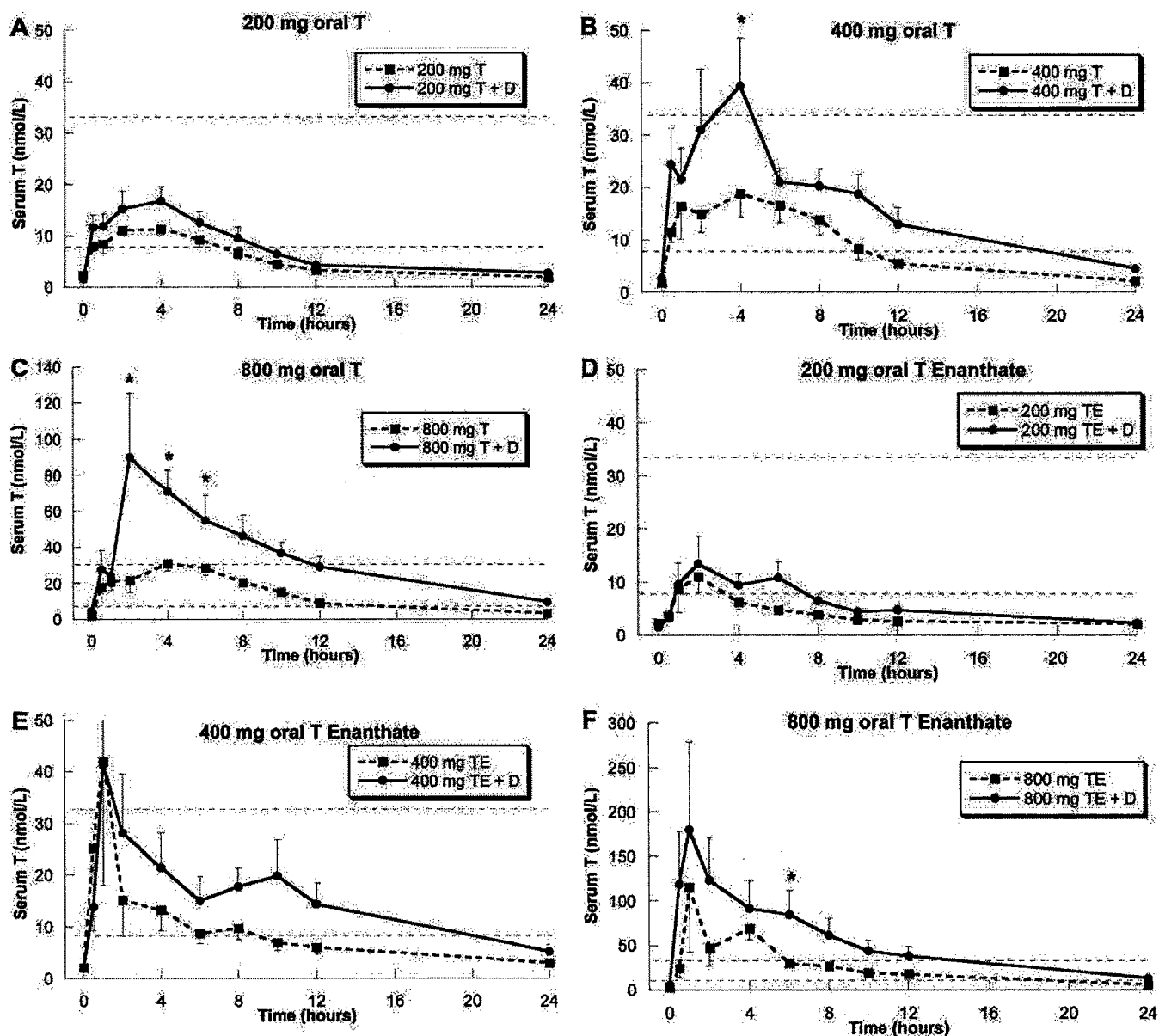


FIG. 2. Serum T concentrations (mean \pm SEM) after oral administration of 200, 400, and 800 mg T in oil (A–C) and TE in oil (D–F) with and without D for 24 h in normal men treated with the GnRH antagonist acyline to temporarily suspend T production. Note the larger y-axis for the 800-mg dose. The dotted lines represent the upper and lower limits of the normal range for serum T. *, $P < 0.05$ compared with T alone.

TABLE 2. T pharmacokinetics after administration of a single dose of oral T and oral TE in oil with and without D to normal men previously administered a GnRH antagonist

| Testosterone (n = 7) | T Only | | | T + D | | |
|----------------------|------------|-----------------------|--------------------------|-------------------------|----------------------------|--------------------------|
| | 200 mg | 400 mg | 800 mg | 200 mg | 400 mg | 800 mg |
| Cmax (nmol/liter) | 12.3 ± 4.1 | 26.1 ± 15.1 | 40.4 ± 10.1 ^a | 22.2 ± 8.4 ^b | 50.3 ± 30.9 ^{a,b} | 122.1 ± 82 |
| Tmax (h) | 2.8 ± 1.9 | 3.9 ± 2.6 | 3.1 ± 2.0 | 3.1 ± 2.0 | 3.8 ± 3.1 | 3.4 ± 1.5 |
| AUC (nmol-h/liter) | 124 ± 28 | 208 ± 74 ^a | 328 ± 72 ^a | 176 ± 46 ^c | 393 ± 140 ^{a,c} | 846 ± 363 ^{a,c} |
| t _{1/2} (h) | 10.4 ± 2.9 | 10.7 ± 6.0 | 8.1 ± 5.0 | 9.9 ± 3.8 | 9.0 ± 2.8 | 7.8 ± 3.2 |

| TE (n = 6) | TE only | | | TE + D | | |
|----------------------|------------|-----------|------------------------|------------|------------------------|-------------|
| | 200 mg | 400 mg | 800 mg | 200 mg | 400 mg | 800 mg |
| Cmax (nmol/liter) | 14.6 ± 8.5 | 51.8 ± 59 | 160.8 ± 149 | 20.2 ± 9.4 | 74 ± 55 ^a | 229 ± 228 |
| Tmax (h) | 3.2 ± 2.6 | 4.1 ± 4.0 | 2.7 ± 1.5 | 4.1 ± 4.2 | 4.3 ± 3.8 | 3.3 ± 2.4 |
| AUC (nmol-h/liter) | 90 ± 27 | 200 ± 140 | 612 ± 249 ^d | 141 ± 41 | 450 ± 196 ^a | 1327 ± 1021 |
| t _{1/2} (h) | 10 ± 2.4 | 10 ± 3.2 | 8.4 ± 3.2 | 9.4 ± 3.2 | 9.2 ± 2.9 | 8.4 ± 2.4 |

Values are the mean ± SD. AUC, Area under the curve; Cmax, maximum concentration after dosing; Tmax, time of maximum concentration.

^a $P < 0.05$ vs. immediately lower dose.

^b $P < 0.05$ vs. T and TE only.

^c $P < 0.01$ vs. T only.

each dose of T were not significantly different from those 24 h after acylone administration.

With the administration of both oral T and oral TE in oil, serum T was significantly increased in a dose-dependent fashion (Fig. 2; $P < 0.01$ for trend). In addition, the maximum concentrations of T, average concentrations of serum T, and area under the curve of serum T increased significantly in a dose-dependent fashion (Table 2 and Fig. 3A), with the maximum concentration of T after oil dosing exceeding the normal range for the 800-mg dose of T and the 400- and 800-mg doses of oral TE in oil. The time of maximum concentration was between 2.5 and 4.5 h in all cases, and the calculated terminal $t_{1/2}$ of oral T and TE in oil was between 7.5 and 11 h.

Coadministration of D with oral T or TE in oil significantly

increased the resulting serum T levels compared with administration of T or TE alone (Fig. 2; $P < 0.01$ for trend). The maximum concentration of T after oral treatment with the combination of T or TE and D exceeded the normal range for both the 400- and 800-mg doses of T and TE in oil. Similar to the administration of T or TE only, the time to maximum concentration remained between 2.5 and 4.5 h, and the calculated terminal $t_{1/2}$ was between 8 and 10 h. The T area under the curve for the combination of T and D was significantly increased at all doses compared with that for T alone [200 mg, 124 ± 28 nmol-h/liter (T alone) vs. 176 ± 45 nmol-h/liter (T + D); 400 mg, 208 ± 74 nmol-h/liter (T alone) vs. 393 nmol-h/liter (T plus D); 800 mg, 328 ± 82 nmol-h/liter (T alone) vs. 846 ± 363 nmol-h/liter (T plus D); $P < 0.01$ for all comparisons].

Serum DHT levels

Serum DHT decreased significantly 24 h after acylone administration (d 0 DHT, 1.6 ± 0.6 nmol/liter; d 1 DHT, 0.6 ± 0.2 nmol/liter; $P < 0.05$). There was no difference in serum DHT levels 24 h after acylone administration between groups (T, 0.5 ± 0.2; TE, 0.6 ± 0.2; $P = 0.63$).

The administration of both oral T and oral TE in oil significantly increased serum DHT in a dose-dependent fashion (Fig. 4). In addition, the maximum concentration of DHT and the area under the curve increased significantly (Table 3), with the maximum concentration of DHT after oral treatment exceeding the normal range for all doses of T and TE in oil. The time of maximum concentration was between 3.9 and 6 h in all cases, and the calculated terminal $t_{1/2}$ of oral T and TE in oil was between 7.5 and 11 h.

Coadministration of D with oral T or TE in oil significantly decreased both maximum and average serum DHT levels compared with the administration of T or TE alone (Fig. 3B and Table 3). The maximum concentration of DHT after oral treatment with the combination of T and D exceeded the normal range at the 800-mg dose of T and at the 400- and 800-mg doses of TE in oil. The time to maximum concentration was between 2.5 and 7.5 h, and the calculated terminal $t_{1/2}$ was between 8 and 10 h. The DHT area under the curve

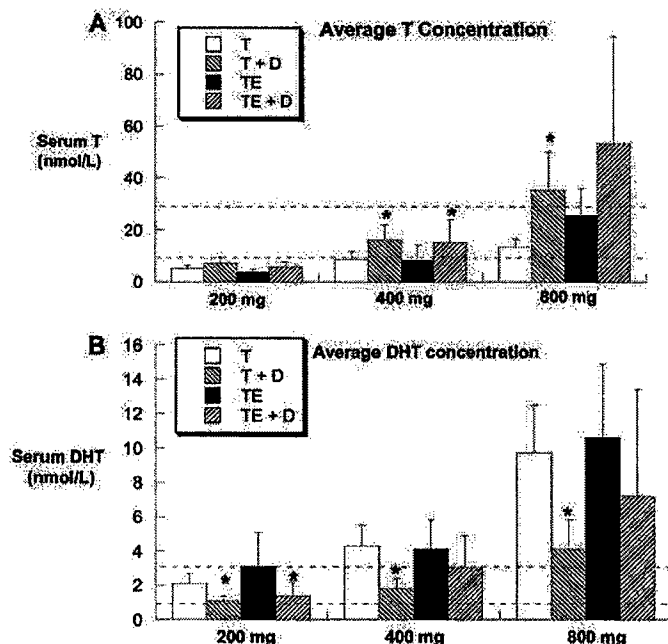


FIG. 3. Average serum T (A) and DHT (B) concentrations (mean ± SD) over the 24-h interval after oral treatment. The dotted lines represent the upper and lower limits of the normal range for serum T. *, $P < 0.05$ compared with T alone.

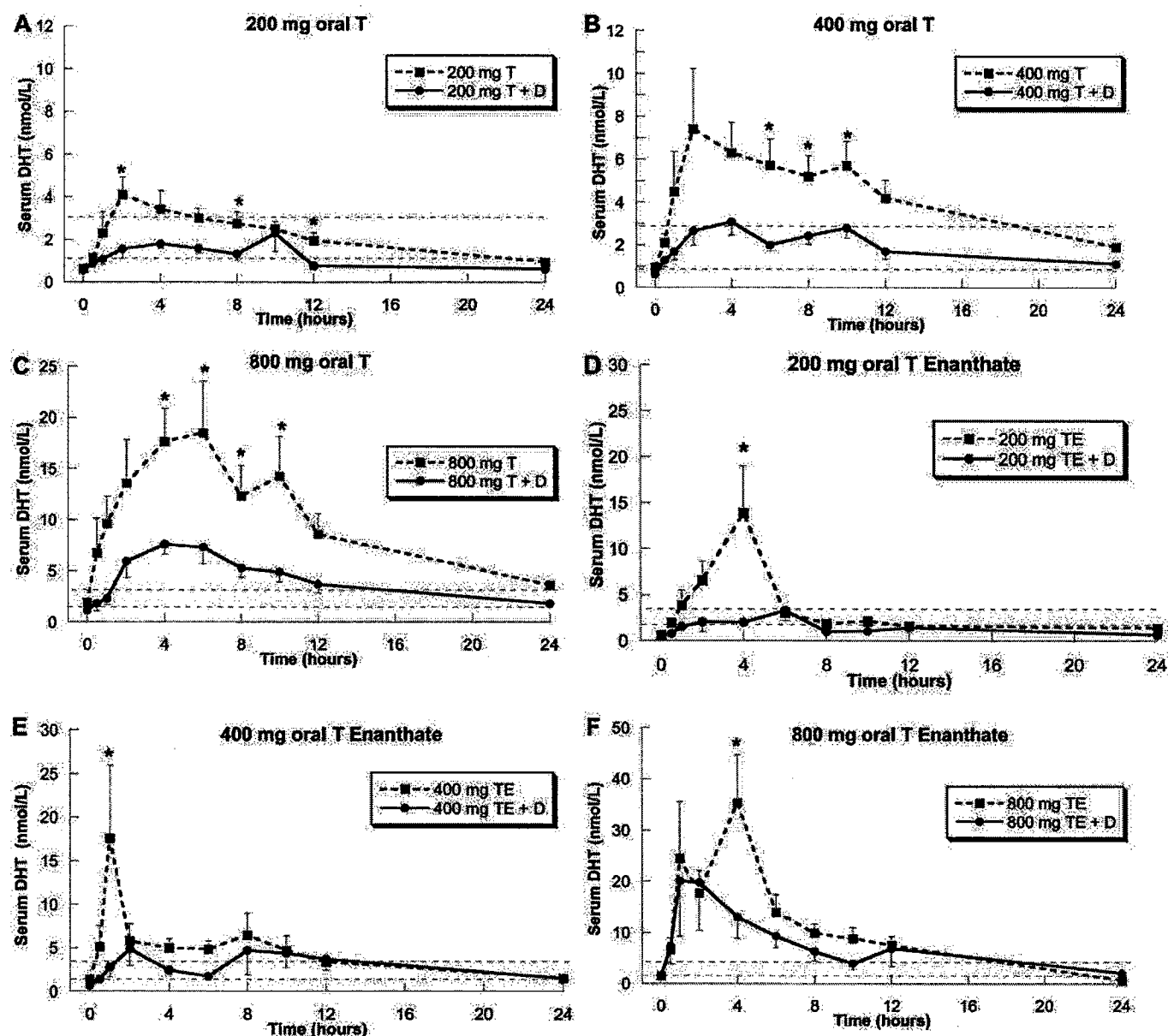


FIG. 4. Serum DHT concentrations (mean \pm SEM) after oral administration of 200, 400, and 800 mg T in oil (A–C) and TE in oil (D–F) with and without D for 24 h in normal men treated with the GnRH antagonist acyline to temporarily suspend T production. Note the larger y-axis for the 800-mg dose. The dotted lines represent the upper and lower limits of the normal range for serum DHT. *, $P < 0.05$ compared with T plus D.

for the combination of T and D was significantly decreased compared with the area under the curve for T alone at all doses.

Serum E2 and SHBG

Mean serum E2 levels were not significantly different between the treatment groups on d 0 [134 ± 21 (T) vs. 116 ± 30 (TE) pmol/liter] or 24 h after acyline administration [94 ± 14 (T) vs. 87 ± 12 (TE) pmol/liter]. With oral administration of T or TE, serum E2 levels increased nonsignificantly compared with baseline levels with the 800-mg dose in both the T and TE groups (Fig. 5), but all E2 levels remained within the normal range. There were no significant differences in

serum E2 between either T or TE alone compared with T or TE with D coadministration. Serum SHBG did not change significantly after administration of acyline or oral administration of T or TE in oil either with or without concomitant D administration (Fig. 6).

Discussion

In this study we have demonstrated that single doses of T or TE when administered orally in oil can result in serum T levels that would be useful for the treatment of T deficiency. Secondly, we have demonstrated that addition of the 5 α -reductase inhibitor D to oral T in oil 1) significantly increases the serum T levels achieved after a given dose of T, and 2)

TABLE 3. DHT pharmacokinetics after administration of a single dose of oral T and oral TE in oil with and without D to normal men previously administered a GnRH antagonist

| T (n = 7) | T Only | | | T + D | | |
|-------------------------------|-----------|-------------------------|-------------------------|------------------------|--------------------------|---------------------------|
| | 200 mg | 400 mg | 800 mg | 200 mg | 400 mg | 800 mg |
| C _{max} (nmol/liter) | 5.6 ± 2.0 | 12.0 ± 3.9 ^a | 30.0 ± 7.0 ^a | 2.2 ± 0.7 ^b | 4.2 ± 1.6 ^{a,b} | 10.3 ± 3.5 ^{a,b} |
| T _{max} (h) | 4.7 ± 3.4 | 5.0 ± 3.8 | 3.9 ± 3.5 | 5.1 ± 3.0 | 6.0 ± 3.3 | 4.6 ± 2.2 |
| AUC (nmol-h/liter) | 51 ± 15 | 106 ± 29 ^a | 239 ± 71 ^a | 25 ± 8.5 ^c | 45 ± 15 ^{a,b} | 99 ± 40 ^{a,b} |
| t _{1/2} (h) | 10 ± 2.3 | 9.3 ± 2.0 | 7.5 ± 3.6 | 9.9 ± 3.8 | 10.6 ± 2.3 | 9.9 ± 2.2 |

| TE (n = 6) | TE only | | | TE + D | | |
|-------------------------------|-----------|-----------|--------------------------|------------------------|------------------------|------------------------|
| | 200 mg | 400 mg | 800 mg | 200 mg | 400 mg | 800 mg |
| C _{max} (nmol/liter) | 15.3 ± 12 | 21.0 ± 19 | 48.8 ± 22.6 ^a | 4.0 ± 2.4 ^c | 8.0 ± 6.5 ^a | 25.3 ± 24 ^c |
| T _{max} (h) | 3.2 ± 1.3 | 4.2 ± 3.5 | 2.5 ± 1.6 | 5.5 ± 3.7 | 7.2 ± 4.0 | 2.7 ± 2.0 |
| AUC (nmol-h/liter) | 75 ± 48 | 100 ± 42 | 253 ± 101 | 35 ± 18 ^c | 72 ± 45 | 173 ± 148 |
| t _{1/2} (h) | 10 ± 2.2 | 9.1 ± 3.5 | 8.6 ± 2.9 | 8.6 ± 3.5 | 8.4 ± 3.4 | 9.0 ± 3.3 |

Values are the mean ± SD. AUC, Area under the curve; C_{avg}, average concentration during 24-h period after dosing; C_{max}, maximum concentration after dosing; T_{max}, time of maximum concentration.

^a *P* < 0.05 vs. immediately lower dose.

^b *P* < 0.01 vs. T only.

^c *P* < 0.05 vs. T and TE only.

attenuates the supraphysiological elevations in serum DHT seen with the administration of oral T or T esters (e.g. TU) without concomitant 5 α -reductase inhibition.

These data contradict the prevailing wisdom in the field, which states that the oral route for T delivery is impractical

due to near-complete hepatic first-pass metabolism of orally administered T (11). Although it is true that the bioavailability of orally administered T is very low, probably around 1% (30, 31), our work demonstrates that if sufficient T is administered orally in oil, potentially therapeutic levels of serum T can be achieved after oral dosing. It is likely that liver metabolism of orally dosed T is extensive, because oral T administered to men with cirrhosis results in serum T levels that are markedly elevated compared with normal controls (32, 33). Whether long-term administration of oral T in oil would induce increased hepatic metabolism of oral T and therefore reduce T bioavailability will be the subject of future research.

Previous studies of the oral administration of T may have found reduced levels of serum T in part due to 5 α -reductase activity in the intestine and liver (34). In this study using T or TE, and in the work of others with TU (24–27), serum levels of DHT after oral administration are markedly elevated, implying that a large fraction of the orally adminis-

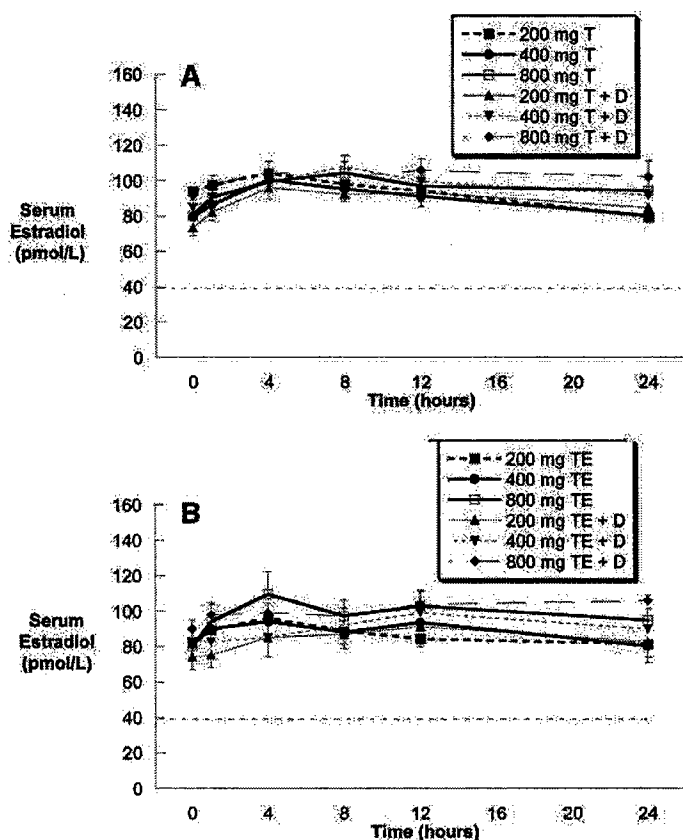


FIG. 5. Serum E2 (mean ± SEM) after oral administration of 200, 400, and 800 mg T (A) and TE (B) in oil with and without D for 24 h in normal men treated with the GnRH antagonist acyline to temporarily suspend T production. The dotted line represents the lower limit of the normal range.

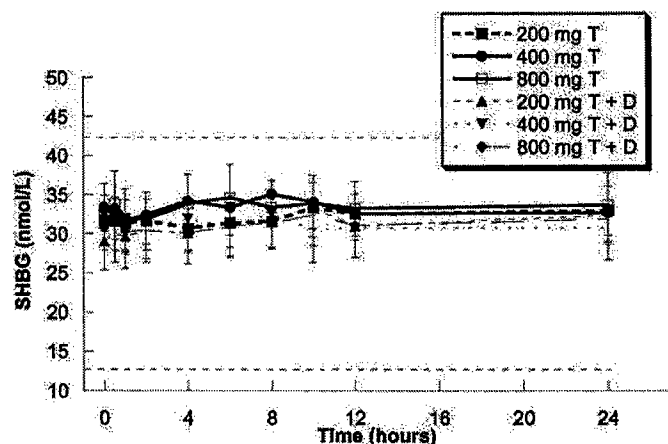


FIG. 6. Serum SHBG concentrations (mean ± SEM) after oral administration of 200, 400, and 800 mg T and TE in oil with and without D for 24 h in normal men treated with the GnRH antagonist acyline to temporarily suspend T production. The dotted lines represent the upper and lower limits of the normal range.

tered T dose may be metabolized in the liver and intestines to DHT. Surprisingly, in this study, the coadministration of a 5 α -reductase inhibitor roughly doubles the average T concentration and the area under the curve for the serum T while reducing the elevations of serum DHT by approximately half. These marked elevations in serum T with concomitant 5 α -reductase inhibition are probably due to inhibition of the 5 α -reductase enzyme in intestine and liver, which appears to account for approximately one half of the metabolism of T after an oral dose. Importantly, the combination of elevated serum T without marked elevations in serum DHT may allow for selective oral androgen therapy, which may be useful in decreasing the risk for DHT-dependent disease, such as benign prostate hyperplasia and prostate cancer.

It is also important to note that previous studies of oral T administration demonstrating poor oral bioavailability of T have used T in powder form at doses of 100 and 200 mg (21–23). We have tested oral T in powder form in doses as high as 400 mg without achieving therapeutic serum T levels (data not shown), implying that the administration of T in oil is crucial for the achievement of the therapeutic serum T levels seen in this study. It has been previously shown that the absorption of oral TU is markedly affected by concomitant intake of fatty foods (27, 30). This is probably due to the fact that much of the orally administered TU is absorbed via the lymphatics (35). In an animal model of TU absorption, more than 80% of the bioavailable T is thought to be absorbed via the lymphatics (36). Whether food intake will affect the absorption of oral T in oil is unknown and probably depends on how much of the dose is absorbed via lymphatics *vs.* via the portal circulation. Because T was administered in oil in this study, some of the dose may have been absorbed via the lymphatics. This might explain in part the unexpectedly long serum half-life of T seen with oral compared with iv administration of T, which has been reported to have a half-life of less than 1 h (31, 37). Another possibility is that there is some degree of enterohepatic circulation of the orally administered T, prolonging the apparent half-life in serum. Because of this uncertainty, the impact of food intake on the absorption and serum levels of T after the administration of oral high dose T will be the subject of future study.

It is important to note that there was no evidence of either liver or kidney toxicity associated with the doses of oral T administered in this study; however, additional long-term study of these doses combined with a 5 α -reductase inhibitor will be required to determine the safety of this approach to T therapy. Although one subject did report transient gynecomastia, this subject's serum E2 level remained within the normal range. Additionally, no subject complained of impotence, decreased libido, or sexual dysfunction during the treatment period. These side effects have been reported when D is administered alone for benign prostate hyperplasia (38); however, in theory, they would be less likely when D is administered in combination with T. Additionally, the implication of long-term 5 α -reductase inhibition will need examination given the increase in high grade prostate cancer (despite an overall decrease in prostate cancer incidence) seen with chronic finasteride administration in the prostate cancer prevention trial (39).

There were slight, nonsignificant increases in serum E2

seen after oral dosing of T and TE in oil. This implies that although orally administered T can undergo aromatization to E2, it does not do so at high levels, suggesting that there is probably little aromatase activity in the intestine and liver in man. This finding is reassuring in showing that orally administered T is likely to allow for the important functions of estrogen in man, such as maintenance of bone density (40), but not lead to an increased risk of estrogen-related side effects such as gynecomastia.

From a practical standpoint, a regimen using oral T in oil in the formulation used in this study may need to be administered twice daily; however, additional refinements of this approach, such as the use of slow-release capsules, may allow for more controlled release of T in the intestine and could lead to a formulation that could be administered orally once daily, a major improvement over current T replacement options.

In conclusion, we have demonstrated that single doses of T or TE, when administered orally in oil, can result in markedly elevated serum levels of T in normal men with induced hypogonadism; such levels would presumably be therapeutically effective in treating testicular failure. In addition, we have demonstrated that addition of the 5 α -reductase inhibitor D to oral T in oil significantly increases the serum T levels observed with a given dose of T and attenuates the supraphysiological elevations in serum DHT seen with the administration of oral T alone. Combinations of oral T and 5 α -reductase inhibitors may allow for an oral, selective form of androgen therapy. Additional studies of the long-term safety, pharmacokinetics, and pharmacodynamics of this combination are warranted to determine whether it might be a clinically useful and attractive method of treating T deficiency.

Acknowledgments

We acknowledge Ms. Amanda Wiseman for assistance with study implementation, and Ms. Dorothy McGuinness for performing the hormone assays.

Received June 25, 2004. Accepted February 9, 2005.

Address all correspondence and requests for reprints to: Dr. John K. Amory, University of Washington, Box 356429, 1959 NE Pacific Street, Seattle, Washington 98195. E-mail: jamory@u.washington.edu.

This work was supported by the National Institute of Child Health and Human Development, a division of the National Institutes of Health, through Cooperative Agreements U54-HD-12629 and U54-HD-42454 as part of the specialized Cooperative Centers Program in Reproductive Research and the Cooperative Contraceptive Research Centers Program. J.K.A. is supported in part by the National Institute of Child Health and Human Development, a division of the National Institutes of Health (NIH), by Grant 1K23-HD-45386-10A1. A portion of this work was conducted through the Clinical Research Center facility at University of Washington and supported by NIH Grant M01-RR-00037.

References

1. Kelch RP, Jenner MR, Weinstein R, Kaplan SL, Grumbach MM 1972 Estradiol and testosterone in the male: secretion by human, simian, and canine testes. *J Clin Invest* 51:824–830
2. Weinstein R, Kelch RP, Jenner MR, Kaplan SL, Grumbach MM 1974 Secretion of androgens and estrogens by the normal and abnormal human testis. *J Clin Invest* 53:1–6
3. Plymate SR 2001 Male hypogonadism. In: Becker KL, ed. *Principles and practice of endocrinology and metabolism*, 3rd Ed. Philadelphia: Lippincott Williams & Wilkins; 1125–1150
4. Katznelson L, Finkelstein JS, Schoenfeld DA, Rosenthal KI, Klibanski A 1996 Increase in bone density and lean body mass during testosterone ad-

- ministration in men with acquired hypogonadism. *J Clin Endocrinol Metab* 81:4358–4365
5. Behre HM, Kliesch S, Leifke R, Link RM, Nieschlag E 1997 Long-term effect of testosterone therapy on bone mineral density in hypogonadal men. *J Clin Endocrinol Metab* 82:2386–2390
 6. Bhasin S, Bremner WJ 1997 Emerging issues in androgen replacement therapy. *J Clin Endocrinol Metab* 1982:3–8
 7. Wang C, Alexander G, Berman N, Salehian B, Davidson T, McDonald V, Steiner B, Hull L, Callegari C, Swerdloff RS 1996 Testosterone replacement therapy improves mood in hypogonadal men—a clinical research center study. *J Clin Endocrinol Metab* 81:3578–3583
 8. Snyder PJ, Peachey H, Berlin JA, Hannoush P, Haddad G, Dlewati A, Santanna J, Loh L, Lenrow DA, Holmes JH, Kapoor SC, Atkinson LE, Strom BL 2000 Effects of testosterone replacement in hypogonadal men. *J Clin Endocrinol Metab* 85:2670–2677
 9. Bagatell CJ, Bremner WJ 1996 Androgens in men—uses and abuses. *N Engl J Med* 334:707–714
 10. Matsumoto AM 2001 Clinical use and abuse of androgens and antiandrogens. In: Becker KL, ed. *Principles and practice of endocrinology and metabolism*, 3rd Ed. Philadelphia: Lippincott Williams & Wilkins; 1181–1199
 11. Snyder P 2001 Androgens. In: Hardman JG, Lumbard LE, eds. *Goodman and Gilman's the pharmacological basis of therapeutics*, 4th Ed. New York: McGraw-Hill; 1635–1648
 12. Westaby D, Ogle SJ, Paradinis FJ, Randell JB, Murray-Lyon IM 1977 Liver damage from long-term methyltestosterone. *Lancet* 2:262–263
 13. Turani H, Levi J, Zevin D, Kessler E 1983 Hepatic lesions in patients on anabolic androgenic therapy. *Isr J Med Sci* 19:332–337
 14. Lowdell CP, Murray-Lyon IM 1985 Reversal of liver damage due to long term methyltestosterone and safety of non-17- α alkylated androgens. *Br Med J* 291:637–645
 15. Cabasso A 1994 Peliosis hepatitis in a young adult bodybuilder. *Med Sci Sports Exerc* 26:2–4
 16. Pavlatos AM, Fultz O, Monberg MJ, Vootkur A 2001 Review of oxymetholone: a 17- α -alkylated anabolic-androgenic steroid. *Clin Ther* 23:789–801
 17. Fossa SD, Opjordsmoen S, Haug E 1999 Androgen replacement and quality of life in patients treated for bilateral testicular cancer. *Eur J Cancer* 35:1220–1225
 18. Amory JK, Matsumoto AM 1998 The therapeutic potential of testosterone patches. *Exp Opin Invest Drugs* 7:1977–1985
 19. Swerdloff RS, Wang C, Cunningham G, Dobs A, Iranmanesh A, Matsumoto AM, Snyder PJ, Weber T, Longstreth J, Berman N 2000 Long-term pharmacokinetics of transdermal testosterone gel in hypogonadal men. *J Clin Endocrinol Metab* 85:4500–4507
 20. Foss GL 1939 Clinical administration of androgens. *Lancet* 1:502–504
 21. Nieschlag E, Mauss J, Coert A, Kicovic P 1975 Plasma androgen levels in men after oral administration of testosterone and testosterone undecanoate. *Acta Endocrinol (Copenh)* 79:366–374
 22. Johnsen SG, Bennett EP, Jensen VG 1974 Therapeutic effectiveness of oral testosterone. *Lancet* 2:1473–1475
 23. Daggett PR, Wheeler MJ, Nabarro JDN 1978 Oral testosterone, a reappraisal. *Horm Res* 9:121–129
 24. Skakkebaek NE, Bancroft J, Davidson DW, Warner P 1981 Androgen replacement with oral testosterone undecanoate in hypogonadal men: a double-blind controlled study. *Clin Endocrinol (Oxf)* 14:49–61
 25. Gooren LJG 1994 A ten-year safety study of the oral androgen testosterone undecanoate. *J Androl* 15:212–215
 26. Houwing NS, Maris F, Schnabel PG, Bagchus WM 2003 Pharmacokinetic study in women of three different doses of a new formulation of oral testosterone undecanoate, Andriol Testocaps. *Pharmacotherapy* 23:1257–1265
 27. Bagchus WM, Hust R, Maris F, Schnabel PG, Houwing NS 2003 Important effect of food on the bioavailability of oral testosterone undecanoate. *Pharmacotherapy* 23:319–325
 28. Roehrborn CG, Boyle P, Nickel JC, Hoefner K, Andriole G 2002 Efficacy and safety of a dual inhibitor of 5 α -reductase types 1 and 2 (dutasteride) in men with benign prostatic hyperplasia. *Urology* 60:434–441
 29. Herbst KL, Coviello AD, Page S, Amory JK, Anawalt BD, Bremner WJ 2004 A single dose of the potent gonadotropin-releasing hormone antagonist acyl-line suppresses gonadotropins and testosterone for 2 weeks in healthy young men. *J Clin Endocrinol Metab* 89:5959–5965
 30. Frey H, Aakvaag A, Saanum D, Falch J 1979 Bioavailability of oral testosterone in males. *Eur J Clin Pharmacol* 16:345–349
 31. Tauber U, Schroder K, Dusterberg B, Matthes H 1986 Absolute bioavailability of testosterone after oral administration of testosterone undecanoate and testosterone. *Eur J Drug Metab Pharmacokinet* 11:145–149
 32. Glud C, Bennett P, Dietrichson O, Johnsen SG, Ranek L, Swendsen LB, Juhl E 1981 Short-term parenteral and peroral testosterone administration in men with alcoholic cirrhosis. *Scand J Gastroenterol* 16:749–755
 33. Glud C, Bahnsen M, Bennett P, Dietrichson O, Henriksen JH, Johnsen SG, Swendsen LB, Brodthagen UA, Juhl E 1983 Oral testosterone load related to liver function in men with alcoholic cirrhosis. *Scand J Gastroenterol* 18:391–396
 34. Thigpen AE, Silver RJ, Guileyardo JM, Casey ML, McConnell JD, Russell DW 1993 Tissue distribution and ontogeny of steroid 5 α reductase isozymes expression. *J Clin Invest* 92:903–910
 35. Horst HJ, Holtje WJ, Dennis M, Coert A, Geelen J, Voigt KD 1976 Lymphatic absorption and metabolism of orally administered testosterone undecanoate in man. *Klin Wschr* 54:875–879
 36. Shackelford DM, Faassen WA, Houwing N, Lass H, Edwards GA, Porter CJ, Charman WN 2003 Contribution of lymphatically transported testosterone undecanoate to the systemic exposure of testosterone after oral administration of two andriol formulations in conscious lymph duct-cannulated dogs. *J Pharmacol Exp Ther* 306:925–933
 37. Southren AK, Gordon GG, Tochimoto S, Pinzon G, Lane DR, Stypulkowski W 1967 Mean plasma concentration, metabolic clearance and basal testosterone production rates of testosterone in normal young men and women using a constant infusion procedure: effect of time of day and plasma concentration on the metabolic clearance rate of testosterone. *J Clin Endocrinol* 27:686–694
 38. Andriole GL, Kirby R 2003 Safety and tolerability of the dual 5- α -reductase inhibitor dutasteride in the treatment of benign prostatic hyperplasia. *Eur Urol* 44:82–88
 39. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, Minasian LM, Ford LG, Lippman SM, Crawford ED, Crowley JJ, Coltman Jr CA 2003 The influence of finasteride on the development of prostate cancer. *N Engl J Med* 349:215–224
 40. Khosla S, Melton LJ, Riggs BL 2001 Estrogens and bone health in men. *Calcif Tissue Int* 69:189–192

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

Exogenous Testosterone or Testosterone with Finasteride Increases Bone Mineral Density in Older Men with Low Serum Testosterone

JOHN K. AMORY, NELSON B. WATTS, KIRK A. EASLEY, PAUL R. SUTTON,
BRADLEY D. ANAWALT, ALVIN M. MATSUMOTO, WILLIAM J. BREMNER, AND J. LISA TENOVER

Departments of Medicine (J.L.T.) and Biostatistics (K.A.E.), Emory University School of Medicine, Rollins School of Public Health, Atlanta, Georgia 30329; Department of Medicine (N.B.W.), University of Cincinnati College of Medicine, Cincinnati, Ohio 45267; and Department of Medicine (J.K.A., P.R.S., B.D.A., A.M.M., W.J.B.), Veterans Affairs-Puget Sound Health Care System (B.D.A. and A.M.M.), and Geriatric Research, Education and Clinical Center (A.M.M.), University of Washington School of Medicine, Seattle, Washington 98195

Older men, particularly those with low serum testosterone (T) levels, might benefit from T therapy to improve bone mineral density (BMD) and reduce fracture risk. Concerns exist, however, about the impact of T therapy on the prostate in older men. We hypothesized that the combination of T and finasteride (F), a 5 α -reductase inhibitor, might increase BMD in older men without adverse effects on the prostate. Seventy men aged 65 yr or older, with a serum T less than 12.1 nmol/liter on two occasions, were randomly assigned to receive one of three regimens for 36 months: T enanthate, 200 mg im every 2 wk with placebo pills daily (T-only); T enanthate, 200 mg every 2 wk with 5 mg F daily (T+F); or placebo injections and pills (placebo). Low BMD was not an inclusion criterion. We obtained serial measurements of BMD of the lumbar spine and hip by dual x-ray absorptiometry. Prostate-specific antigen (PSA) and prostate size were measured at baseline and during treatment to assess the impact of therapy on the prostate. Fifty men completed the 36-month protocol. By an intent-to-treat analysis including all men for as long as they contributed data, T therapy for 36 months increased BMD in these men at the lumbar spine [$10.2 \pm 1.4\%$ (mean percentage increase from baseline \pm SEM; T-only) and $9.3 \pm 1.4\%$ (T+F) vs. $1.3 \pm 1.4\%$ for placebo ($P < 0.001$)] and in the hip [$2.7 \pm 0.7\%$ (T-only) and $2.2 \pm 0.7\%$ (T+F) vs. $-0.2 \pm 0.7\%$ for placebo, ($P \leq 0.02$)]. Significant

increases in BMD were seen also in the intertrochanteric and trochanteric regions of the hip. After 6 months of therapy, urinary deoxypyridinoline (a bone-resorption marker) decreased significantly compared with baseline in both the T-only and T+F groups ($P < 0.001$) but was not significantly reduced compared with the placebo group. Over 36 months, PSA increased significantly from baseline in the T-only group ($P < 0.001$). Prostate volume increased in all groups during the 36-month treatment period, but this increase was significantly less in the T+F group compared with both the T-only and placebo groups ($P = 0.02$). These results demonstrate that T therapy in older men with low serum T increases vertebral and hip BMD over 36 months, both when administered alone and when combined with F. This finding suggests that dihydrotestosterone is not essential for the beneficial effects of T on BMD in men. In addition, the concomitant administration of F with T appears to attenuate the impact of T therapy on prostate size and PSA and might reduce the chance of benign prostatic hypertrophy or other prostate-related complications in older men on T therapy. These findings have important implications for the prevention and treatment of osteoporosis in older men with low T levels. (*J Clin Endocrinol Metab* 89: 503–510, 2004)

TWENTY PERCENT OF men over age 60 have serum testosterone (T) concentrations below the normal range for young men (1, 2). Because low T levels are associated with an increased risk of osteoporosis and fracture (3–7), T therapy in older men might increase bone mineral density (BMD) and reduce fracture risk. Studies in young, hypogonadal men have demonstrated that T therapy increases BMD (8–11), but few studies have investigated older patients, who are at greater risk of fracture. Two randomized clinical trials of transdermal T treatment in men over the age of 64 yr have been published (12, 13). In the first study, vertebral but not hip BMD increased, and only in those with low pretreatment

T levels (12). In the second study, T prevented the loss of hip BMD observed in the placebo-treated men (13). Therefore, significant questions still exist about the ability of T therapy in older men to have significant impact on bone health.

The relative roles of T and its metabolite, dihydrotestosterone (DHT), in regulating BMD are not clear. Because DHT contributes to the development of benign prostatic hypertrophy (BPH) and possibly prostate cancer, increasing T levels without also increasing DHT might be preferable in older men, especially if DHT has little or no effect on BMD. Finasteride (F) inhibits DHT production by blocking the enzyme 5 α -reductase, which converts T to DHT, and has been used safely to treat BPH in older men without compromising BMD (14–16).

We hypothesized that long-term im T therapy in older men who had serum T below the range of normal for young adult men would significantly increase BMD. Furthermore, we hypothesized that the addition of the 5 α -reductase inhibitor F would have no impact on T-mediated increases in BMD but

Abbreviations: BMD, Bone mineral density; BPH, benign prostatic hypertrophy; CV, coefficient of variation; DHT, dihydrotestosterone; E2, estradiol; F, finasteride; po, per os; PSA, prostate-specific antigen; T, testosterone; TE, T enanthate.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

would minimize the potential for adverse effects on prostate health. Therefore, we conducted a randomized, double-blind, placebo-controlled trial of im T administration, with or without F, to test these hypotheses.

Subjects and Methods

Subjects

Men aged 65 yr and older were recruited using advertisements and direct mailings. The inclusion criterion was a nonfasting, morning serum total T level below 12.1 nmol/liter (350 ng/dl) for 2 d. Exclusion criteria included the following: severe illness; use of medications including anabolic steroids, antiandrogens, glucocorticoids, bisphosphonates, diuretics, calcitonin, seizure medications, or warfarin; Paget's disease; smoking or heavy alcohol use; sleep apnea; hematocrit greater than 48%; total cholesterol above 300 mg/dl; abnormal kidney, liver, thyroid, adrenal, or pituitary function; regular exercise more than three times a week; prostate issues [prostate cancer, a prostate nodule on exam, prostate-specific antigen (PSA) >4.0 ng/ml, or International Prostate Symptom Score >8]; urinary postvoid residual by ultrasound of more than 149 ml; or an abnormal transrectal ultrasound. Reduced BMD was not an inclusion criterion. The Institutional Review Board of Emory University, where all subject interactions occurred, approved the study, and subjects gave written informed consent before screening.

A total of 676 men were evaluated for eligibility. Of these, 283 men were potentially eligible and underwent T measurement. One hundred ten men met the T criterion and underwent further screening tests; 70 men were enrolled. Forty men who passed initial screening were not enrolled for the following reasons: abnormal PSA, prostate ultrasound, postvoid residual, or symptom score (11); pituitary, thyroid, or adrenal disease (5); medical illness (4); second T levels above 350 ng/dl (4); total cholesterol above 300 mg/dl (1); or being eligible but refusing enrollment (15).

Study design

Participants were randomized to one of three treatment groups: 1) T-only group, T enanthate (TE; Schein Pharmaceuticals, Florham Park, NJ) 200 mg im every 2 wk, plus placebo pill orally [*per os* (po)] daily; 2) T+F group, TE 200 mg im every 2 wk, plus F (Merck & Co., Rahway, NJ) 5 mg po daily; or 3) placebo group, sesame oil placebo 1 ml im every 2 wk, plus placebo pill po daily. The estimate of sample size for the trial was based on the percentage change in BMD from baseline to 6-month follow-up. Assuming a clinically important increase on average of 1% in the T+F group, no change on average in the placebo group, and an estimated SD in each group of 1%, a sample size of 17 men per group ensured approximately 80% statistical power to detect a treatment difference of 1% (significance level, 0.05; two-sided test) if the true difference between groups was a 1% BMD increase from baseline to 6-month follow-up. Allowing for a 30% dropout rate over 3 yr, 70 patients were randomized in the trial.

The order of treatment assignment was randomly computer-generated in permuted blocks of six. Participants were treated for 36 months. Only the research pharmacist and safety monitoring board knew of the randomization. A nurse administered the injections, and 98% occurred within 2 d of the scheduled time. There was 95% compliance with the daily F or placebo in the enrolled subjects based on monthly pill counts. The study design included the potential for dose reduction of T or placebo injection by decrements of 0.2 ml (40 mg of TE for subjects actually receiving T) for a hematocrit of more than 52% on safety monitoring performed at 2, 4, 8, 12, 18, 24, and 30 months. Calcium and vitamin D supplements were not provided, but patients were allowed to continue these medications if they were taking them already. Participants were queried at the beginning and end of the study in regard to the intake of these supplements, with no significant change in their use being noted. Specifically, two men in the placebo group, one man in the T-only group, and none in the T+F group were taking calcium supplements during the study. No subject was taking additional vitamin D.

For men who discontinued the study prematurely, telephone follow-up was conducted to ascertain clinical outcomes.

Measurements

At baseline and after 6, 12, 18, 24, and 36 months of treatment, BMD was measured at the lumbar spine (L1-L4; anteroposterior view only) and in the nondominant hip by dual x-ray absorptiometry using a Hologic QDR-2000 densitometer (Hologic, Waltham MA) that was standardized daily. The intraperson coefficient of variation (CV) was 1.0% for both the spine and the hip. T and Z scores were calculated using male databases; the manufacturer's database was used for the spine, and the National Health and Nutrition Examination Survey III was used for all of the hip measurements. One of the investigators (N.B.W.) who was blinded to treatment analyzed all of the BMD measurements and excluded from analysis vertebrae that showed localized degenerative change, compression fractures, or other confounding factors. One or two vertebrae were deleted from analysis if there was obvious degenerative change on the image and/or if it was 1 SD or more higher than the lowest vertebrae (six placebo subjects, five T-only subjects, and five T+F subjects). If three or more vertebrae showed evidence of degenerative change, the spine measurement was considered invalid and was not used (no placebo subjects, two T-only subjects, and one T+F subject).

Blood was drawn for hormone measurements in the morning at baseline and immediately before injections after 2, 4, 6, 8, 12, 18, 24, 30, and 36 months of treatment. Samples at baseline 6, 12, 18, 24, and 36 months were fasting samples, whereas the other samples were nonfasting. Blood was drawn for markers of bone metabolism after a 12-h fast at baseline and after 6 months of treatment. For a subset of men ($n = 22$), additional morning blood was drawn at the end of the first study year on d 3 or 4, 7, and 11 of the T-dosing period to obtain between-nadir samples. Serum samples for 25-hydroxyvitamin D and intact PTH were assayed immediately. All other samples were stored frozen at -70°C until the end of the study, when serum samples from each participant were assayed concurrently. A 2-h morning urine was collected for measurement of deoxypyridinoline at baseline and after 6 months of treatment. T, SHBG, and estradiol (E2) were measured using fluoroimmunoassays (Delfia, Wallac Oy, Turku, Finland). The intraassay and interassay CVs for midrange measurements were 4.5 and 9.5% for T, 4.0 and 11.1% for SHBG, and 3.6 and 6.0% for E2. The normal range is 12–33 nmol/liter for T and 60–220 pmol/liter for E2. DHT was measured by RIA (Endocrine Sciences, Calabasas Hills, CA); the midrange intraassay and interassay CVs were 6.6 and 14%, respectively. Non-SHBG-bound, bioavailable T was assayed using RIA after ammonium sulfate precipitation [Centre Hospitalier de l'Université at Laval University (CHUL) Research Center, Sainte-Foy, Quebec, Canada]; the midrange intraassay and interassay CVs were 7.4 and 12%, respectively. Osteocalcin was measured by RIA (Diagnostic Systems Laboratories, Inc., Webster, TX); the midrange intraassay and interassay CVs were both 7.2%. Bone-specific alkaline phosphatase was measured by immunoassay (Metra Biosystems, Mountain View, CA); the midrange intraassay and interassay CVs were 1.4 and 4.8%, respectively. Urinary deoxypyridinoline was measured by chromatography after acid hydrolysis and was normalized to urinary creatinine; midrange intraassay and interassay CVs were 8 and 15%, respectively. Intact PTH was measured by a chemiluminescent assay (Diagnostic Products Corp., Los Angeles, CA); midrange intraassay and interassay CVs were 5.1 and 5.3%, respectively.

Participant monitoring

Participants were examined monthly. Measurements of hematocrit and transaminases occurred every 2 months for 1 yr, and every 6 months thereafter. Prostate volume was assessed by transrectal ultrasound (Brüel & Kjaer, Borås, Sweden) at baseline (model 3535) and at the end of treatment (model 1846 PM) using established techniques (17, 18). PSA levels were measured every 4 months during the first year and every 6 months thereafter; digital rectal examination was performed every 6 months.

Statistical analysis

The primary analyses of the data were performed according to patients' original treatment assignment (*i.e.* intention-to-treat analyses), and all men were included in the analyses for as long as they contributed data. Baseline characteristics between treatment groups were compared with the Kruskal-Wallis test. Repeated-measures analyses for each of the

four BMD measurements were analyzed as percentage change from baseline with a means model with SAS Proc Mixed (version 8; SAS Institute, Inc., Cary, NC) providing separate estimates of the means by time on the study (6, 12, 18, 24, 30, and 36 months) and treatment groups. An unstructured variance-covariance form among the repeated measurements was assumed for each outcome, and estimates of the SE values of parameters were used to perform statistical tests and construct 95% confidence intervals. Student's *t* tests were used to compare the pairwise differences between the model-based treatment means (least-squares means) at each time point or treatment month. The model-based means are unbiased with unbalanced and missing data, as long as the missing data are noninformative (missing at random). A dropout process is assumed to be missing at random if, depending on the observed data, the dropout is independent of the unobserved measurements. Mean changes over time within a treatment group were tested for linear trend. Repeated-measures analyses were also performed for T, DHT, E2, and PSA after a log transformation, and for prostate volume, hematocrit, hemoglobin, and lipids. The Wilcoxon signed-rank test was used to compare change from baseline to 6 months within each treatment group for six markers of bone metabolism. Statistical tests were two-sided. A Bonferroni adjustment ($P < 0.0167$) was used for the three pairwise comparisons performed at each treatment month.

Results

Seventy men, with a mean age of 71 ± 4 yr (range, 65–83 yr), participated in the study. Twenty-four were randomized to T-only, 22 to T+F, and 24 to placebo. Fifty men completed the entire 36 months of the study. Of the 20 men who did not complete the study, six were in the placebo group, and seven each were in the T-only and T+F groups. Reasons for discontinuation included the following: personal reasons (10 men), intercurrent illness (7 men), or a new diagnosis of

prostate cancer (3 men). At baseline, the three treatment groups did not differ significantly from each other in age, body mass index, hormone levels, BMD, prostate volume, or PSA (Table 1). Twenty-four of the 70 men (10 in the placebo group, 8 in the T-only, and 6 in the T+F group) had baseline serum E2 levels that were below the lower limit of normal. The baseline BMD for the hip and lumbar spine for the participants was similar to that for a standard male population of the same age (Z scores, Table 1). A total of seven men, two each in the placebo and T+F groups and three in the T-only group, had low baseline lumbar-spine BMD (T score more than 2.5 sd below peak bone mass for young men), whereas four men, two in the placebo group and one each in the T-only and T+F groups, had low baseline hip BMD. There were no significant differences in the baseline characteristics between the men who discontinued and those who completed the study. Reduction of T dosage was necessary in 14 men (seven in the T-only group and seven in the T+F group *vs.* none in the placebo group). After the decrease in T dosage, the final mean (\pm sd) dose of TE was 158 ± 36 mg for the T-only group and 164 ± 40 mg for the T+F group every 2 wk.

Mean nadir serum total T, bioavailable T, and E2 levels in the T-only and T+F groups significantly increased throughout the treatment period (Fig. 1, A–C), whereas these hormone levels did not change in the placebo group. Nadir serum total T and E2 levels tended to be higher in the T+F group compared with the T-only group, but this difference

TABLE 1. Baseline characteristics (mean \pm SD) of 70 older men administered im T alone, T with F, or placebo for 36 months

| Characteristic | Placebo (n = 24) | T-only group (n = 24) | T + F group (n = 22) | P |
|--------------------------------------|------------------|-----------------------|----------------------|------|
| Age (yr) | 71 ± 5 | 71 ± 4 | 71 ± 4 | 0.99 |
| Body mass index (kg/m ²) | 27.8 ± 3.6 | 28.7 ± 3.6 | 27.0 ± 2.7 | 0.24 |
| Hormones | | | | |
| Total testosterone (nmol/liter) | 10.5 ± 1.7 | 9.9 ± 1.6 | 10.1 ± 2.1 | 0.36 |
| Dihydrotestosterone (nmol/liter) | 1.0 ± 0.5 | 0.8 ± 0.3 | 0.9 ± 0.2 | 0.48 |
| Estradiol (pmol/liter) | 83.3 ± 44.4 | 71.5 ± 33.7 | 84.0 ± 33.3 | 0.47 |
| SHBG (nmol/liter) | 44.0 ± 18.1 | 45.2 ± 16.6 | 48.2 ± 15.0 | 0.55 |
| Bioavailable T (nmol/liter) | 3.5 ± 1.3 | 3.3 ± 1.2 | 3.4 ± 1.2 | 0.62 |
| BMD | | | | |
| Lumbar spine | | | | |
| Density (g/cm ²) | 1.04 ± 0.15 | 1.06 ± 0.16 | 1.03 ± 0.19 | 0.81 |
| T score ^a | -0.48 ± 1.47 | -0.30 ± 1.56 | -0.53 ± 1.66 | |
| Z score ^a | 0.44 ± 1.49 | 0.69 ± 1.62 | 0.51 ± 1.67 | |
| Total hip | | | | |
| Density (g/cm ²) | 0.96 ± 0.13 | 0.96 ± 0.14 | 0.90 ± 0.23 | 0.67 |
| T score | -0.87 ± 0.89 | -0.84 ± 1.13 | -1.02 ± 0.80 | |
| Z score | 0.30 ± 0.86 | 0.24 ± 1.21 | 0.12 ± 0.79 | |
| Intertrochanter | | | | |
| Density (g/cm ²) | 1.11 ± 0.15 | 1.11 ± 0.17 | 1.11 ± 0.13 | 0.96 |
| T score | -0.90 ± 0.88 | -0.77 ± 1.17 | -0.80 ± 0.96 | |
| Z score | 0.06 ± 1.10 | 0.29 ± 1.21 | 0.16 ± 0.82 | |
| Trochanter | | | | |
| Density (g/cm ²) | 0.74 ± 0.11 | 0.75 ± 0.11 | 0.71 ± 0.10 | 0.29 |
| T score | -0.92 ± 2.32 | -0.39 ± 1.10 | -0.73 ± 0.86 | |
| Z score | 0.27 ± 1.16 | 0.45 ± 1.17 | 0.13 ± 0.85 | |
| Femoral neck | | | | |
| Density (g/cm ²) | 0.81 ± 0.12 | 0.78 ± 0.13 | 0.74 ± 0.09 | 0.19 |
| T score | -1.56 ± 1.07 | -1.77 ± 1.16 | -2.03 ± 0.87 | |
| Z score | 0.42 ± 1.06 | 0.19 ± 1.17 | 0.03 ± 0.86 | |
| Prostate parameters | | | | |
| PSA (ng/dl) | 1.4 ± 1.1 | 0.9 ± 0.8 | 1.0 ± 0.6 | 0.08 |
| Prostate volume (cm ³) | 32 ± 14 | 29 ± 11 | 33 ± 16 | 0.80 |

^a T score compares the BMD to the mean for young normal males and Z score compares it with age-matched controls.

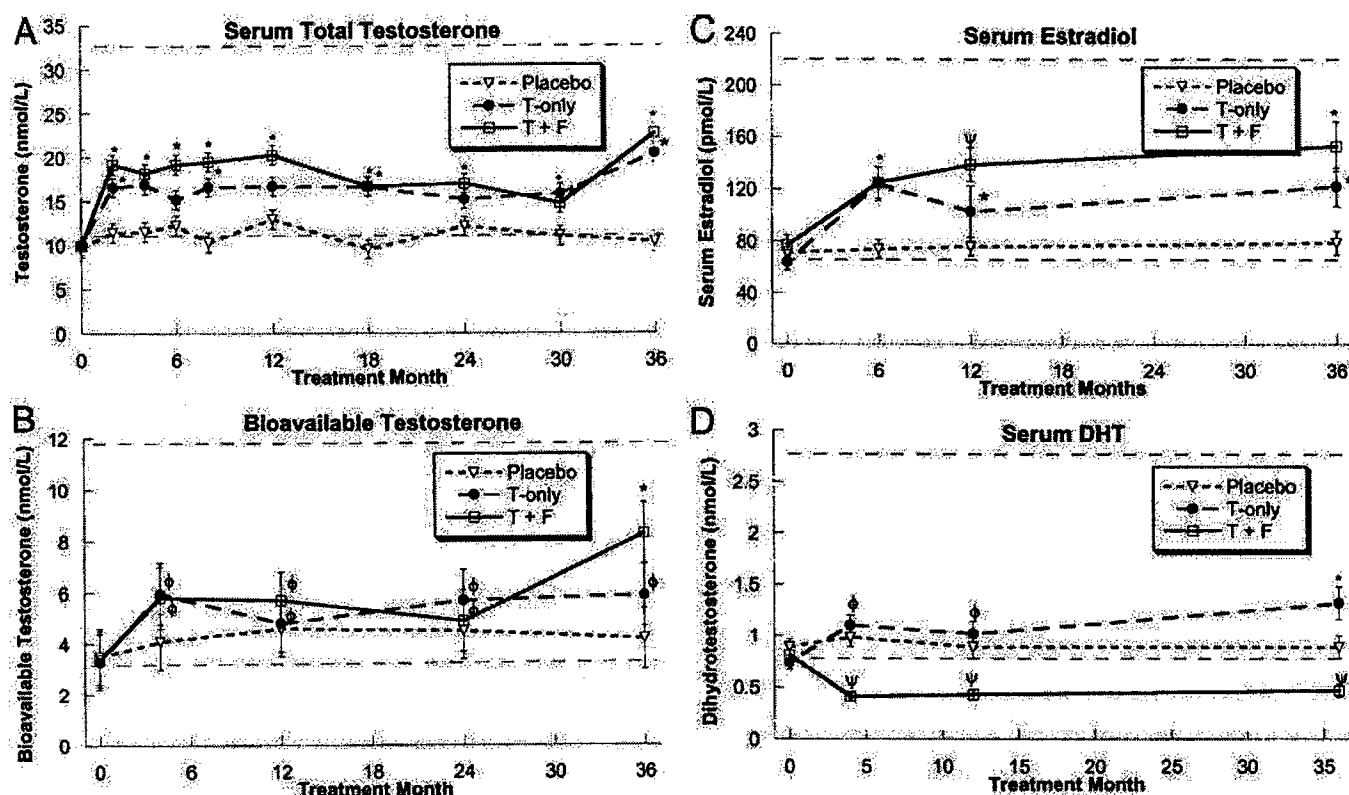


FIG. 1. Geometric mean (\pm SEM) nadir serum total T (A), bioavailable (non-SHBG-bound) T (B), E2 (C), and DHT (D) in older men with low T who were treated with either T (T-only), T and F (T+F), or placebo for 36 months. Horizontal dotted lines represent normal ranges. *, $P < 0.05$ compared with baseline and placebo; ψ , $P < 0.05$ compared with baseline, placebo, and T-only; ϕ , $P < 0.05$ compared with baseline.

did not reach statistical significance for any time point for total T and was only significant at month 12 for E2 ($P = 0.03$). For the subset of men from whom blood was sampled at multiple times throughout the 2-wk T-dosing period (six men in the placebo group, seven men in the T-only group, and nine men in the T+F group), peak serum total T levels were at or above the normal serum T range for the two T treatment groups, with a mean peak value for the T-only group being 35.9 ± 12.1 nmol/liter (mean \pm SD) and that for the T+F group being 43.5 ± 7.6 nmol/liter. Average total T levels during the 2-wk dosing interval were 25.8 ± 6.0 , 33.0 ± 6.4 , and 11.8 ± 2.3 nmol/liter for the T-only, T+F, and placebo groups, respectively. In comparing these two T treatment groups, there was no difference in peak T levels ($P = 0.13$), but the average total T levels were somewhat higher in the T+F group ($P = 0.04$).

Mean nadir serum DHT levels did not change throughout the study in the placebo group, increased significantly in the T-only group, and decreased in the T+F group ($P < 0.001$ compared with baseline and placebo) by 6 months and remained suppressed throughout treatment (Fig. 1D). The maximum decline in serum DHT levels in the T+F group was 50% below baseline, which was reached at treatment month 4.

BMD and metabolism

BMD of the lumbar spine, total hip, and trochanteric and intertrochanteric regions increased in both the T-only and the

T+F groups during the study period, whereas those in the placebo group did not change ($P < 0.001$; Table 2 and Fig. 2, A–D). The mean percentage increase from baseline in BMD of the lumbar spine was significant ($P < 0.001$) for the T-only and T+F groups, but the mean did not change for men in the placebo group ($P = 0.39$ for linear trend). There was no significant change over the 36 months in the BMD at the femoral neck in any of three treatment groups ($P = 0.16$). In the T groups, increases in lumbar BMD were positively correlated with magnitude of increase in both serum total T ($r = 0.44$; $P = 0.001$), bioavailable T ($r = 0.45$ and $P = 0.009$), and serum E2 ($r = 0.45$; $P = 0.0006$) but were not related to baseline BMD; to baseline levels of total T, bioavailable T, DHT, or E2; or to baseline levels of T or E2 after correction for baseline SHBG levels.

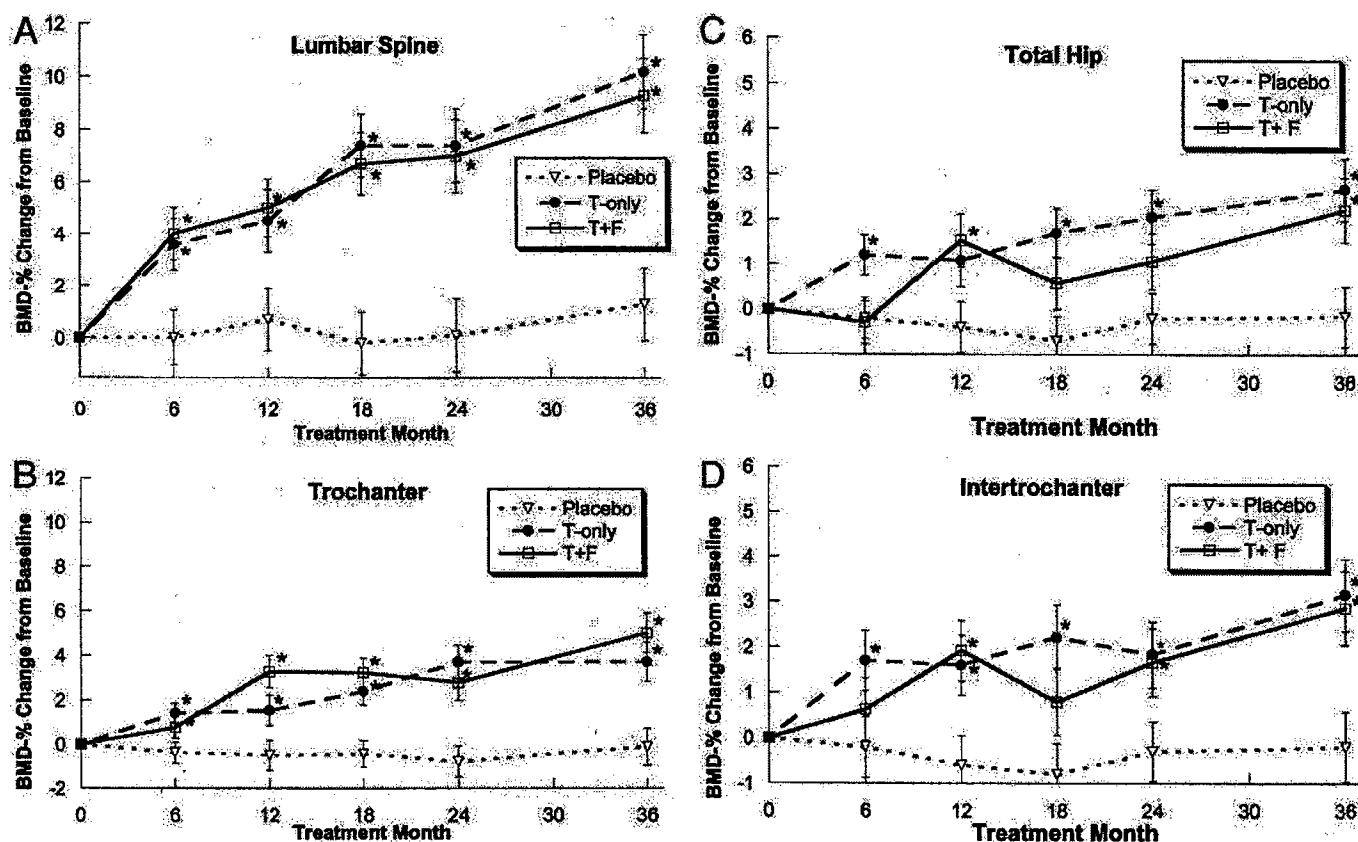
After 6 months of therapy, serum osteocalcin (a bone formation marker), intact PTH, and 25-hydroxyvitamin D did not change in any group (Table 3). In contrast, urinary deoxypyridinoline, a bone resorption marker, decreased significantly in both the T-only and T+F groups (both $P < 0.001$) but was unchanged in the placebo group ($P = 0.30$). Bone-specific alkaline phosphatase, a bone formation marker, decreased significantly in the T-only group ($P = 0.049$).

Prostate and hematological effects

Forty-nine of the 50 subjects who completed the 36-month study underwent end-of-treatment prostate ultrasound, and

TABLE 2. Mean BMD (g/cm^2) and 95% confidence interval measured by dual-energy x-ray absorptiometry in older men administered in T alone, T with F (T+F), or placebo at the lumbar spine, total hip, femoral neck, and intertrochanteric and trochanteric regions^a

| | Baseline | 6 months | 12 months | 18 months | 24 months | 36 months |
|---------------------------------|------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Lumbar spine | | | | | | |
| T only | 1.06 (0.98–1.13) | 1.09 (1.02–1.16) ^b | 1.10 (1.03–1.17) ^b | 1.13 (1.05–1.21) ^b | 1.13 (1.05–1.20) ^b | 1.16 (1.08–1.24) ^b |
| T+F | 1.03 (0.96–1.10) | 1.07 (0.99–1.14) ^b | 1.08 (1.00–1.15) ^b | 1.10 (1.02–1.17) ^b | 1.10 (1.03–1.17) ^b | 1.13 (1.05–1.20) ^b |
| Placebo | 1.04 (0.97–1.11) | 1.05 (0.97–1.12) | 1.05 (0.98–1.13) | 1.04 (0.97–1.12) | 1.05 (0.97–1.12) | 1.06 (0.98–1.14) |
| Total hip | | | | | | |
| T only | 0.96 (0.90–1.00) | 0.97 (0.91–1.02) | 0.96 (0.91–1.02) | 0.97 (0.92–1.03) ^b | 0.97 (0.92–1.03) ^b | 0.98 (0.92–1.04) ^b |
| T+F | 0.94 (0.89–1.00) | 0.94 (0.88–0.99) | 0.96 (0.90–1.01) | 0.95 (0.89–1.01) | 0.95 (0.90–1.01) | 0.96 (0.90–1.02) ^b |
| Placebo | 0.96 (0.90–1.01) | 0.95 (0.90–1.01) | 0.95 (0.90–1.00) | 0.95 (0.90–1.00) | 0.95 (0.91–1.01) | 0.95 (0.90–1.01) |
| Intertrochanteric region | | | | | | |
| T only | 1.11 (1.05–1.17) | 1.13 (1.06–1.19) | 1.13 (1.06–1.19) | 1.13 (1.07–1.20) ^b | 1.13 (1.07–1.20) | 1.15 (1.08–1.21) ^b |
| T+F | 1.11 (1.04–1.17) | 1.11 (1.04–1.18) | 1.13 (1.06–1.19) | 1.11 (1.05–1.18) | 1.12 (1.06–1.19) | 1.14 (1.07–1.21) ^b |
| Placebo | 1.11 (1.04–1.17) | 1.10 (1.04–1.17) | 1.10 (1.04–1.16) | 1.10 (1.03–1.16) | 1.10 (1.04–1.16) | 1.10 (1.04–1.17) |
| Trochanteric region | | | | | | |
| T only | 0.75 (0.70–0.79) | 0.76 (0.71–0.80) ^b | 0.76 (0.71–0.81) | 0.77 (0.72–0.81) ^b | 0.78 (0.73–0.82) ^b | 0.78 (0.73–0.83) ^b |
| T+F | 0.71 (0.66–0.76) | 0.71 (0.67–0.76) | 0.73 (0.68–0.78) ^b | 0.73 (0.68–0.78) ^b | 0.73 (0.68–0.78) ^b | 0.75 (0.69–0.80) ^b |
| Placebo | 0.74 (0.69–0.78) | 0.74 (0.69–0.78) | 0.74 (0.69–0.78) | 0.74 (0.69–0.78) | 0.73 (0.69–0.78) | 0.74 (0.69–0.79) |
| Femoral neck | | | | | | |
| T only | 0.78 (0.73–0.82) | 0.79 (0.74–0.83) | 0.78 (0.74–0.83) | 0.79 (0.74–0.84) ^b | 0.79 (0.74–0.83) | 0.79 (0.74–0.84) ^b |
| T+F | 0.74 (0.69–0.79) | 0.75 (0.70–0.79) | 0.76 (0.71–0.80) | 0.76 (0.71–0.81) | 0.76 (0.71–0.81) | 0.77 (0.72–0.82) ^b |
| Placebo | 0.81 (0.76–0.85) | 0.81 (0.76–0.85) | 0.80 (0.76–0.85) | 0.80 (0.76–0.85) | 0.81 (0.76–0.85) | 0.81 (0.76–0.86) |

^a Includes data from all measurements available at a given time point.^b $P < 0.01$ compared with baseline.**FIG. 2.** Mean percentage increase (\pm SEM) in BMD of the lumbar spine (A), trochanteric (B), total hip (C), and intertrochanteric (D) regions in older men with low T who were treated with either T (T-only), T and F (T+F), or placebo for 36 months. *, $P < 0.05$ compared with baseline and placebo.

all subjects had an end-of-treatment PSA. There was a small but significant increase in serum PSA in the T-only group ($P < 0.001$ by month 36), but there was no change in PSA in either the placebo or T+F group at any time during the study

(Table 4). Prostate volume increased significantly in all groups over the 3-yr study period. The increase in prostate volume in the T-only group was similar to the increase seen in the placebo treatment group ($P = 0.35$), whereas the in-

TABLE 3. Markers of bone metabolism during treatment (median, 75th–25th percentiles) in older men administered im T alone, T with F, or placebo after 6 months of therapy

| | Placebo | | T only | | T+F | |
|-----------------------------------|-------------|-------------|-------------|-------------------------|-------------|------------------------|
| | Baseline | Month 6 | Baseline | Month 6 | Baseline | Month 6 |
| Osteocalcin (ng/ml) | 2.06 (1.79) | 1.79 (2.44) | 2.35 (2.35) | 2.40 (1.37) | 2.38 (2.16) | 2.02 (2.08) |
| BSAP (IU/liter) | 14.3 (4.8) | 14.3 (5.2) | 14.6 (7.5) | 12.0 (5.2) ^a | 16.0 (5.6) | 13.9 (4.7) |
| U-deoxypyridinoline (μmol/mol Cr) | 5.8 (2.0) | 6.1 (2.5) | 6.2 (2.3) | 5.0 (1.6) ^b | 6.0 (2.0) | 4.5 (1.6) ^b |
| PTH (pg/ml) | 45 (28) | 42 (15) | 44 (28) | 50 (40) | 40 (19) | 52 (27) |
| 25 hydroxy-vitamin D (ng/ml) | 34 (20) | 44 (19) | 35 (21) | 39 (24) | 41 (12) | 48 (13) |

BSAP, Bone-specific alkaline phosphatase; U, urinary; PTH, intact PTH; Cr, creatinine.

^a $P < 0.05$ compared to baseline.^b $P < 0.001$ compared to baseline.

crease in prostate volume in the T+F group was significantly less than in the T-only group ($P = 0.02$).

One man in the placebo group, two in the T-only group, and no subjects in the T+F group had a diagnosis of prostate cancer, leading to discontinuation of study participation ($P = 0.46$). The two men with prostate cancer in the T-only group were diagnosed after 7 and 8 months of study participation. The indication for biopsy in one case was an abnormal digital rectal exam and in the other case was asthenia and fever. In both cases, one of six biopsy samples revealed Gleason grade 5 disease. For the man with the abnormal digital rectal exam, the positive biopsy occurred in the opposite lobe from the abnormal exam finding. The individual in the placebo group with prostate cancer was diagnosed after 24 months in the study; the indication for biopsy was an elevated PSA. Of the 17 remaining men who discontinued participation in the study, 15 were free of prostate cancer or other prostate diseases at the conclusion of the study. Two men were lost to follow-up.

Mean hematocrit and hemoglobin values increased significantly during treatment in the T-only and T+F groups ($P < 0.001$ compared with baseline and placebo) but were unchanged in the placebo group (Table 4). Increase in hematocrit was positively associated with elevations in T ($r = 0.41$; $P < 0.001$). In the T-only group, one man suffered a cerebral hemorrhage during treatment, and another man developed new symptoms of sleep apnea confirmed by a sleep study. There were no other serious adverse cardiovascular, cerebrovascular, or pulmonary events.

Discussion

This study demonstrates that im T therapy in older men with low serum total T levels increases BMD in the lumbar spine and hip over 3 yr. The increase in BMD would be expected to decrease fracture risk. The increases in BMD seen in this study are similar in magnitude both to those observed with T therapy in younger hypogonadal men (8–11) and to those seen with bisphosphonate therapy in men with osteoporosis (19, 20). The increases seen in BMD were not limited to the spine but also involved most areas of the hip that were measured. Previous studies of T therapy in older men have reported either smaller increases in lumbar spine BMD (12) or no increase in hip BMD with T therapy (12, 13). There could be a number of reasons why the findings of this study differ from the findings of these previous studies. For one, the men enrolled in this study all had baseline serum total T levels that were below the normal range for young men,

which was not the case in one of the previous studies (12). In addition, the serum levels of total T and E2 achieved with im T injections in this study were two to three times higher than those achieved in studies using T patches in older men (12, 13). Such large increases over baseline T and E2 levels might account for much of the difference between the increases in BMD seen in these studies. Although the dose-response range for bone in regard to T (or E2) in older men has not been established yet, these data may suggest that a certain threshold of serum T (or E2) must be reached and/or a certain magnitude of change from baseline levels must be achieved before significant effects of the sex steroids on bone are achieved in older men. It also is important to note that, in contrast to previous studies in older men, subjects in our study were not administered supplemental calcium and vitamin D. This may have increased the magnitude of the differences between placebo and treatment groups we observed in our study; however, dramatic improvements in BMD were seen without calcium and vitamin D supplementation. Whether supplementation in combination with T would result in even greater increases in BMD should be the subject of future research.

Both the T-only and the T+F groups had similar increases in serum nadir total T and E2 levels and in BMD; however, there was a significant decrease in serum DHT in the T+F group. This suggests that conversion to DHT is not essential for the effect of T on BMD. Because F incompletely blocks the conversion of T to DHT (21) and men in our study achieved at best a 50% reduction in serum DHT levels, it is still possible that low levels of DHT are required for stimulating increases in BMD.

The beneficial effects of T therapy on BMD may be mediated by its conversion to E2. The increases in E2 serum levels from baseline with T therapy in this study were substantial. The impact of E2 on BMD in men has been demonstrated in a man with aromatase deficiency who had high serum T levels but low BMD. Treatment with E2 resulted in epiphyseal closure and increased BMD (22). Furthermore, a second man with an E2 receptor mutation was found to have unfused epiphyses and low BMD (23). Other work has suggested that bioavailable E2 may be the best predictor of BMD in older men (24, 25). Although it is likely that E2 plays a major role in maintenance of BMD in men, further studies using nonaromatizable androgens will be required before we will completely understand relative roles of T and E2 in bone formation in men.

The mechanism by which androgens and/or estrogens

TABLE 4. Hematocrit, hemoglobin, prostate size, and PSA during treatment [means (95% confidence interval)]

| | Placebo | | | T-only | | | T+F | | |
|----------------------------------|------------------|------------------|-------------------------|------------------|-------------------------------|-------------------------------|------------------|-------------------------------|-------------------------------|
| | Baseline | Month 18 | Month 36 | Baseline | Month 18 | Month 36 | Baseline | Month 18 | Month 36 |
| Hematology | | | | | | | | | |
| Hematocrit (%) | 43.5 (42.3–44.8) | 42.7 (41.7–44.5) | 42.9 (41.5–44.4) | 42.5 (41.2–43.8) | 48.9 (47.4–50.4) ^a | 48.6 (47.1–50.0) ^a | 43.2 (41.8–44.5) | 48.2 (46.3–49.7) ^a | 47.4 (46.0–49.0) ^a |
| Hemoglobin (g/dl) | 14.7 (14.3–15.2) | 14.5 (14.2–15.2) | 14.6 (14.1–15.1) | 14.5 (14.1–14.9) | 16.5 (16.1–17.0) ^a | 16.6 (16.1–17.1) ^a | 14.6 (14.2–15.1) | 16.6 (16.1–17.0) ^a | 16.2 (15.7–16.7) ^a |
| Prostate | | | | | | | | | |
| Prostate size (cc ^b) | 32 (26–38) | ND | 42 (36–49) ^a | 29 (23–34) | ND | 43 (37–49) ^a | 33 (27–38) | ND | 38 (32–44) ^{a, b} |
| PSA (ng/ml) | 1.4 (1.1–1.9) | 1.5 (1.0–2.1) | 1.7 (1.2–2.3) | 1.0 (0.7–1.3) | 1.4 (1.0–2.0) ^a | 1.4 (1.0–2.0) ^a | 1.0 (0.7–1.3) | 0.8 (0.5–1.0) | 1.1 (0.8–1.6) |

ND, Not done. PSA is expressed as the geometric mean.

^a $P < 0.01$ compared with baseline.^b $P = 0.02$ compared with placebo and T-only.

improve BMD is unclear, but androgen receptors have been identified in osteoblasts (26). In our study, most markers of bone formation were unchanged, but the most sensitive marker of bone resorption (27), urinary deoxypyridinoline, decreased significantly. This suggests that T therapy reduces bone resorption more than it increases bone formation. This finding is in agreement with a recently published study in younger men (28). This “antiresorptive” effect of T also might be mediated by E2 (29) and is a main mechanism by which E2 is thought to increase BMD in postmenopausal women (30).

Regarding the prostate, all groups showed increases in prostate volume that were greater than those observed previously in prospective studies of older men (31, 32). The change in ultrasound equipment between baseline and the end of the study may have contributed to the seemingly larger-than-expected magnitude of volume change between baseline and end-of-study. However, because this would have affected equally all treatment groups, the relative volume change differences seen between treatment groups should still be valid. Notably, the T+F group had significantly less increase in prostate volume than either the T-only or placebo groups. The attenuation of prostate volume enlargement seen in this study with the concomitant use of a 5 α -reductase inhibitor, rather than a reduction in prostate volume that is usually reported with such therapy (32), is mirrored by the less-than-expected reduction in serum DHT levels in the T+F group and may have occurred because of the high serum T levels produced by the T-injection regimen used. Nonetheless, this attenuation of prostate growth by 5 α -reductase inhibition might be important in preventing symptomatic BPH and possibly reducing the risk of prostate cancer in older men treated with long-term T therapy; however, our trial lacked sufficient numbers of subjects to detect any possible benefit of F on the risk of these outcomes. Notably, the recently published prostate cancer prevention trial showed a 25% reduction in new cases of prostate cancer in older men treated with F therapy (33). Clearly, larger studies of T therapy with 5 α -reductase inhibitors in older men will be required before a small increased risk of prostatic complications can be excluded.

Subjects in our study did have a higher rate of erythrocytosis than seen in previous trials of T administration in older men using transcutaneous patches (10, 11). Thirty percent of subjects receiving 200 mg of TE every other week in our study developed a hematocrit greater than 52% and required a reduction in the T dose to an average of 158 mg. This finding is probably due to the high serum T levels, especially peak T levels, that were produced in this study and is consistent with rates of erythrocytosis seen in other studies in which older males have been treated with im T (34). Therefore, it is possible that a dose of 150 mg, rather than 200 mg, of TE every 2 wk might be a safer dosage in older men to prevent problematic erythrocytosis; however, there are not data to demonstrate that this dose will prevent bone loss. It is important to note that no ischemic strokes, heart attacks, or episodes of thromboembolism were observed in our study; however, this study lacked sufficient power to rule out a small increase in such events.

In summary, we conclude that T therapy in older men with

low serum T levels markedly increases BMD in both the spine and the hip over 3 yr. The addition of F to T does not diminish increases in BMD but does decrease prostate growth and increases in PSA compared with treatment with either T alone or placebo. Given its beneficial effects on BMD, larger, long-term randomized studies of T therapy with and without inhibitors of 5 α -reductase should be conducted to better define the risks and benefits of T therapy and its impact on the risk of osteoporotic fractures in older men.

Acknowledgments

We thank Arlen Sarkissian and Dorothy McGuinness for performing hormone assays and Robin Allen for clinical assistance.

Received June 30, 2003. Accepted October 20, 2003.

Address all correspondence and requests for reprints to: Dr. J. Lisa Tenover, Wesley Woods Health Center, 1841 Clifton Road Northeast, Atlanta, Georgia 30329-5102.

This work was supported by National Institutes of Health Grant AG10975.

References

- Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR 2001 Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. *J Clin Endocrinol Metab* 86:724–731
- Feldman HA, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviello AD, Bremner WJ, McKinlay JB 2002 Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts Male Aging Study. *J Clin Endocrinol Metab* 87:589–598
- Stanley HL, Schmitt BP, Poses RM, Deiss WP 1991 Does hypogonadism contribute to the occurrence of minimal trauma hip fracture in elderly men? *J Am Geriatr Soc* 39:766–771
- Jackson JA, Riggs MW, Spiekerman AM 1992 Testosterone deficiency as a risk factor for hip fractures in men: a case-control study. *Am J Med Sci* 304:4–8
- Abbasi AA, Rudman D, Wilson CR, Drinka PJ, Basu SN, Mattson DE, Richardson TJ 1995 Observations on nursing home residents with a history of hip fracture. *Am J Med Sci* 310:229–234
- Onghiphadhanakul B, Rajatanavin R, Chailurkit L, Piaseu N, Teerarungsikul K, Sirisriro R, Komindr S, Puavilai G 1995 Serum testosterone and its relation to bone mineral density and body composition in normal males. *Clin Endocrinol (Oxf)* 43:727–733
- Kenny AM, Prestwood KM, Marcello KM, Raisz LG 2000 Determinants of bone density in healthy older men with low testosterone levels. *J Gerontol A Biol Sci Med Sci* 55:M492–M497
- Katznelson L, Finkelstein JS, Schoenfeld DA, Rosenthal DI, Anderson EJ, Klibanski A 1996 Increase in bone mineral and lean body mass during testosterone administration in men with acquired hypogonadism. *J Clin Endocrinol Metab* 81:4358–4365
- Behre HM, Kliesch S, Leifke E, Link TM, Nieschlag E 1997 Long-term effect of testosterone therapy on bone mineral density in hypogonadal men. *J Clin Endocrinol Metab* 82:2386–2390
- Snyder PJ, Peachey H, Berlin JA, Hannoush P, Haddad G, Dlewati A, Santanna J, Loh L, Lenrow DA, Holmes JK, Kapoor SC, Atkinson LE, Strom BL 2000 Effects of testosterone replacement in hypogonadal men. *J Clin Endocrinol Metab* 85:2670–2677
- Wang C, Swerdloff RS, Iranmanesh A, Dobs S, Snyder PJ, Cunningham B, Matsumoto AM, Weber T, Berman N 2001 Effects of transdermal testosterone gel on bone turnover markers and bone mineral density in hypogonadal men. *Clin Endocrinol (Oxf)* 54:739–750
- Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Holmes JH, Dlewati A, Staley J, Santanna J, Kapoor SC, Attie MF, Haddad Jr JG, Strom BL 1999 Effect of testosterone treatment on bone mineral density in men over 65 years of age. *J Clin Endocrinol Metab* 84:1966–1972
- Kenny AM, Prestwood KM, Gruman CA, Marcello KM, Raisz LG 2001 Effects of transdermal testosterone on bone and muscle in older men with low bioavailable testosterone levels. *J Gerontol A Biol Sci Med Sci* 56:M266–M272
- Matzkin H, Chen J, Weisman Y, Goldray D, Pappas F, Jaccard N, Braf Z 1992 Prolonged treatment with finasteride (a 5 α -reductase inhibitor) does not affect bone density and metabolism. *Clin Endocrinol (Oxf)* 37:432–436
- Tollin SR, Rosen HN, Zurofski K, Saltzman B, Zeind AJ, Berg S, Greenspan SL 1996 Finasteride therapy does not alter bone turnover in men with benign prostatic hyperplasia—a Clinical Research Center study. *J Clin Endocrinol Metab* 81:1031–1034
- Matsumoto AM, Tenover L, McClung M, Mobley D, Geller J, Sullivan M, Grayhack J, Wessells H, Kadmon D, Flanagan M, Zhang GK, Schmidt J, Taylor AM, Lee M, Waldstreicher J, Pless Study Group 2002 The long-term effect of specific type II 5 α -reductase inhibition with finasteride on bone mineral density in men: results of a 4-year placebo controlled trial. *J Urol* 167:2105–2108
- Bosch JK, Hop WC, Niemer QH, Bangma CH, Kirkels WJ, Schroder FH 1994 Parameters of prostate volume and shape in a community based population of men 55 to 74 years old. *J Urol* 152:1501–1504
- Coakley FV, Hricak H 2000 Radiologic anatomy of the prostate gland: a clinical approach. *Radiol Clin North Am* 38:15–30
- Orwoll E, Ettinger M, Weiss S, Miller P, Kendler D, Graham J, Adams S, Weber K, Lorenc R, Pietschmann P, Vandormael K, Lombardi A 2000 Alendronate for the treatment of osteoporosis in men. *N Engl J Med* 343:604–610
- Ringe JD, Faber H, Dorst A 2001 Alendronate treatment of established primary osteoporosis in men: results of a 2-year prospective study. *J Clin Endocrinol Metab* 86:5252–5255
- Rittmaster RS 1997 5 α -Reductase inhibitors. *J Androl* 18:582–587
- Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. *N Engl J Med* 337:91–95
- Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med* 331:1056–1061
- Khosla S, Melton LJ, Atkinson EJ, O'Fallon WM, Klee GG, Riggs BL 1998 Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. *J Clin Endocrinol Metab* 83:2266–2274
- Khosla S, Melton LJ, Atkinson EJ, O'Fallon WM 2001 Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. *J Clin Endocrinol Metab* 86:3555–3561
- Colvard DS, Eriksen EF, Keeting PE, Wilson EM, Lubahn DB, French FS, Riggs BL, Spelsberg TC 1989 Identification of androgen receptors in normal human osteoblast-like cells. *Proc Natl Acad Sci USA* 86:854–857
- Ju HJ, Leung S, Brown B, Stringer MA, Leigh S, Scherrer C, Shepard K, Jenkins D, Knudsen J, Cannon R 1997 Comparison of analytical performance and biological variability of three bone resorption assays. *Clin Chem* 43:1570–1579
- Leder BS, LeBlanc KM, Schoenfeld DA, Eastell R, Finkelstein JS 2003 Differential effects of androgens and estrogens on bone turnover in normal men. *J Clin Endocrinol Metab* 88:204–210
- Falahati-Nini A, Riggs BL, Atkinson EJ, O'Fallon WM, Estell R, Khosla S 2000 Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men. *J Clin Invest* 106:1553–1560
- Barrett-Conner E 1997 Hormone replacement therapy. *BMJ* 317:457–461
- Williams AM, Simon I, Landis PK, Moser C, Christen-Barry W, Carter HB, Metter EJ, Partin AW 1999 Prostatic growth rate determined from MRI data: age-related longitudinal changes. *J Androl* 20:474–480
- McConnell JD, Bruskewitz R, Walsh P, Andriole G, Bieber M, Holtgrewe HL, Albertsen P, Roehrborn CG, Nickel JC, Wang DZ, Taylor AM, Waldstreicher J 1998 The effect of finasteride on the risk of acute urinary retention and the need for surgical treatment among men with benign prostatic hyperplasia. Finasteride Long-term Efficacy and Safety Study Group. *N Engl J Med* 338:557–563
- Thompson IM, Goodman PF, Tangen CM, Lucia MS, Miller GJ, Ford LG, Liever MM, Cespedes RD, Atkins JN, Lippman SM, Carlin SM, Ryan A, Szczepanek CM, Crowley JJ, Coltman Jr CA 2003 The influence of finasteride on the development of prostate cancer. *N Engl J Med* 349:215–224
- Hajjar RR, Kaiser FE, Morley JE 1997 Outcomes of long-term testosterone replacement in older hypogonadal males: a retrospective analysis. *J Clin Endocrinol Metab* 82:3793–3796

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.



WORLD ANTI-DOPING AGENCY

INDEPENDENT OBSERVER REPORT

TOUR DE FRANCE 2003

TABLE OF CONTENTS

| | |
|--|-----------|
| 1. INTRODUCTION | 4 |
| 2. INITIAL PREPARATIONS AND MEETINGS..... | 4 |
| 3. MEDICAL CHECK-UP | 6 |
| A. BLOOD TESTS | 6 |
| B. MEDICAL EXAMINATION | 9 |
| 4. OUT-OF-COMPETITION TESTING BEFORE THE START..... | 10 |
| 5. IN-COMPETITION TESTING | 11 |
| A. SELECTION PROCESS | 13 |
| B. NOTIFICATION | 13 |
| C. ESCORTS..... | 14 |
| D. TESTING AREA | 14 |
| E. PROCEDURES FOR TAKING SAMPLES | 14 |
| F. TRANSPORT | 16 |
| 6. OUT-OF-COMPETITION TESTING DURING THE TOUR | 19 |
| 7. LABORATORY | 20 |
| 8. HANDLING THE RESULTS | 27 |
| A. WHILE THE OBSERVERS WERE PRESENT | 27 |
| B. AFTER THE OBSERVERS' DEPARTURE | 27 |
| 9. INFORMING AND EDUCATING ATHLETES | 30 |
| 10. UCI MEDICAL COMMISSION | 31 |
| 11. MISCELLANEOUS..... | 32 |
| 12. CONCLUSIONS | 32 |
| 13. ACKNOWLEDGEMENTS | 34 |

| | |
|--|-----------|
| 14. MEMBERS OF THE INDEPENDENT OBSERVERS TEAM | 34 |
| 15. APPENDICES | 36 |
| APPENDIX 1: PROCEDURE FOR DETERMINING BLOOD PARAMETERS (UCI) | 36 |
| APPENDIX 2: MEASUREMENT PROTOCOL (UCI) | 38 |
| APPENDIX 3: INFORMATION FOR RIDERS (UCI) | 39 |
| APPENDIX 4: REQUEST FOR INFORMATION 04/08/03 | 40 |
| APPENDIX 5: UCI REPLY (07/08/03)..... | 41 |
| APPENDIX 6: UCI REPLY (06/08/03)..... | 42 |
| APPENDIX 7: FAX TO THE CPLD (18/08/03)..... | 43 |
| APPENDIX 8: REPLY FROM THE CPLD (18/08/03)..... | 44 |
| APPENDIX 9: ASO INFORMATION LEAFLET FOR RIDERS | 45 |
| APPENDIX 10: TOUR DE FRANCE CODE OF ETHICS | 47 |

1. INTRODUCTION

This report is submitted by the team of Independent Observers (IO) from the World Anti-Doping Agency (WADA), which was present at the Tour de France 2003. The IO team was able to observe the anti-doping programme implemented by the various organisations working in cooperation during the Tour: The International Cycling Union (UCI), the French Ministry of Sport, the Amaury Sport Organisation (ASO), the French Council for the Prevention and Fight against Doping (CPLD) and the French National Drug Testing Laboratory (LNDD).

The team appointed by WADA to carry out this task comprised 3 members, all regarded as experts in their particular fields.

2. INITIAL PREPARATIONS AND MEETINGS

In the run up to the 2003 Tour, WADA circulated an agreement among the relevant parties in order to confirm that, in accordance with the mandate of the IO programme, the observers would have access to all the relevant documentation and would be able to observe the anti-doping control process implemented for the Tour de France at its various levels. In spite of a few initial problems associated with the legal constraints specific to the Code of Public Health in France hosting the Tour, it was possible to reach an agreement authorizing the WADA team to observe the following procedures:

- selection procedures;
- notification of the cyclists selected for the controls;
- analysis of samples at the LNDD;
- preparation of the controls;
- compiling of the appropriate forms after the controls;
- preparing the sample for dispatch to the laboratory;
- procedures in the event of a "B" sample.

In addition to this, the observers were able to enter the doping control station, provided that they were medical doctors and there was adequate room to accommodate the cyclist, the doctor taking the sample, the UCI delegate and possibly the accompanying person of the cyclist.

An anonymous copy of the doping control forms, as well as a copy of the analysis reports were also supplied to the observers.

Before the observers arrived in Paris, arrangements were made for several meetings to be held in order to ensure, on the one hand, that just as events were starting off, all the parties concerned would be informed of each other's role in the process, while also ensuring that there would be no problems from an organisational point of view.

Two important meetings were held during the first two days, on 1st and 2nd July, in Paris. During the first meeting between a representative from WADA and M. D. Baal, Deputy Director of the Tour, the entire logistics for the event, including accommodation, transportation, etc. were reviewed and coordinated. A car and driver were made available to the observers from the time they arrived in Paris.

A second meeting took place between representatives from the French Ministry of Sport, CPLD, UCI, ASO, LNDD and the WADA observers, which focused on this first contact being made and on explaining the WADA observers' mission, their objectives and role during a sporting event. All the steps involved in the anti-doping test process implemented during the Tour were described in detail and explained. Furthermore, an agreement was reached on guaranteeing anonymity with regard to the copies of reports sent to the observers.

It is important to note that during the meeting the CPLD expressed its opinion about the exchange of information with the observers relating to the doping control forms. It did not agree with the compromise offered by the French Ministry of Sport and consequently, the CPLD stated that it declined all responsibility in the event of any dispute.

During the meeting the President of the IO team emphasised that this type of mission is carried out based on a positive, constructive approach. These objectives can only be achieved with the cooperation of all the present parties based on a system of ongoing communication to help avoid any problems during the mission.

During this meeting, the President also gave all the relevant authorities copies of the documents guaranteeing confidentiality with regard to the information gathered as part of the mission and to the commitment to expose any personal conflict of interest.

On the evening of 4th July, the WADA observers were invited to attend the general reception for the teams being held at Paris City Hall. Race officials not only made the most of this occasion to inform the teams about the Tour de France's Code of Ethics, along with its regulations on behaviour and safety, but they also made the cyclists and their backup teams aware of the issue of doping¹. The anti-doping testing process was quickly explained and an appeal was made to the cyclists for maintaining the spirit of fair play.

RECOMMENDATIONS

It is essential that in future, agreements between the various parties are reached and signed well beforehand in order to guarantee the best possible organisation for all the parties concerned. The agreement was sent to the relevant parties on 16th May 2003 and it was only on 27th June, virtually just before the observers were due to arrive that agreement was successfully reached.

3. MEDICAL CHECK-UP

Before the start, all the cyclists underwent a medical check-up, including a blood test and a medical examination.

a. BLOOD TESTS

All 198 riders officially registered for the 2003 Tour de France underwent a blood test the day before the start of the race.

The samples were taken at the hotels where the teams were staying from 7.30 AM onwards. These tests were carried out under the responsibility of the UCI, which appointed several teams made up of a doctor to take the sample and two UCI commissioners.

¹ See pages 30-31 of this report for more details

Three samples were taken from each rider: an A sample and a B sample for evaluating the levels of haematocrit, haemoglobin and the percentage of reticulocytes and free plasma haemoglobin; a third sample for evaluating other biological parameters (transaminases, glucose, iron, transferrin, ferritin, cortisol, etc.).

The IO team members observed the procedures for taking the blood samples, compiling the forms and transporting the samples to a temporary laboratory located in a hotel. There were two different teams working in the laboratory. One was from the Lausanne Laboratory and the other from the Ghent Laboratory. Each team was made up of a Scientific Director and a Technician. The Swiss team used a Sysmex ® analyzer, while the Belgian team used a Coulter ACT 8 ®. The laboratory was also equipped with a centrifuge and a Hemocue® analyzer for measuring free plasma haemoglobin. This is the first occasion where the UCI has not only evaluated the usual parameters (haematocrit, haemoglobin and the percentage of reticulocytes), but also free plasma haemoglobin, which rises quite significantly when synthetic haemoglobin is administered.

When the analyses, which were carried out immediately as soon as the samples arrived, showed abnormal profiles (abnormal values or trends), the UCI questioned the rider (or his doctor) about the source of this abnormality, or let him know that he would have another test carried out during the race and that he would be classified as being suspect by the UCI Anti-doping Commission.

The UCI authorised the observers to attend all the health check procedures, but not when the results were being issued. The reason behind that being that the health checks were not part of the anti-doping process. Nevertheless, the observers do not share this view because on several occasions during the Tour, the UCI carried out tests in competition and out of competition based on suspect results from blood tests taken as part of the health checks.

This strategy paid off with a out-of-competition control during the Tour, which showed a positive result for erythropoietin (EPO).

Since this year, the UCI has been using a new protocol for identifying riders with an abnormal blood profile. If, for instance, a rider has a haematocrit value of 48% (below 50%), but if the average value of the four previous samples taken was 43%, the Anti-doping Commission will automatically make this rider provide a urine sample to be screened for EPO.

The two doctors included in the IO team were able to view the UCI's blood parameters database and listen to the explanations from Mario Zorzoli, the UCI doctor, about the strategy linked to the health checks.

Appendices I and II describe the procedure for the medical examination carried out to determine blood parameters and the protocol for measuring free plasma haemoglobin. Appendix III contains the letter sent to all the riders before the race containing some information about the blood tests.

The third blood sample was dispatched in refrigerated form to a laboratory in Switzerland for an evaluation of the other parameters, which would be passed on to the UCI doctors at a later stage.

All the technical and administrative procedures were carried out quickly, in a highly professional manner and with the excellent cooperation of all the riders.

During the health checks the commissioners requested health booklets from all the riders for them to photocopy. During the technical meeting on 4th July the UCI Medical Commission returned the health booklets and informed the team representatives of the results from the health checks.

One member of the IO team was also present at the health checks carried out on 9th July, where all the riders in the six teams were examined.

During the Tour the UCI carried out other health checks after the observers' departure, where all the riders with suspect results had to undergo a out-of-competition control or a control on the same day at the end of the stage in order to screen for erythropoietin.

The day before the first health check, the UCI held a meeting attended by the doctors involved in taking the samples and the laboratory managers to explain to them their duties and responsibilities and to issue the relevant documentation and equipment to them.

The observers would like to congratulate the UCI for implementing this strategy involving health checks, which is still at the moment and was in the past a very important measure for protecting the riders' health. The introduction of testing for abnormal biological profiles and the evaluation of free plasma haemoglobin demonstrate that the UCI strives to improve its health check procedures.

Based on the analysis of the individual assessments, it is possible to divide the riders into three categories:

- Those who will be banned from starting due to a haematocrit level above 50% and a haemoglobin level higher than 17 g/dl;
- Those with no biological abnormalities;
- Those who will be authorised to start but, due to a biological profile regarded as "suspect", will have to be included in the group of riders obliged to take a urine test to be screened for EPO at the end of the prologue.

RECOMMENDATIONS

- *During any future WADA observation mission, the UCI should supply the results from the health checks to the WADA team to prove the system's effectiveness (total, indisputable transparency).*
- *The UCI should provide a copy of the completed form with the sample codes for each rider.*
- *Samples should be transported in refrigerated form in a sealed case, along with an appropriately completed security document.*

b. MEDICAL EXAMINATION

On 3rd and 4th July all the riders underwent a medical examination organised by the medical team employed by the ASO led by Dr. Gérard Porte.

The medical team's aim was to make an initial contact with all the riders. It comprised six doctors and two nurses.

A medical record was completed for each cyclist, who underwent several observations and examinations: weight, height, spirometry, cardiopulmonary auscultation, blood pressure and ECG.

The technical procedure used for measuring weight and height was not appropriate, nor were the conditions ideal for cardiopulmonary auscultation (the rooms in the large hall of the Palais des Expositions did not have integrated ceilings and there was background noise. The cars following the Tour were also located nearby). The cyclists' privacy was not respected either. For instance, the ECGs were carried out in a large room with four beds and no curtains separating them from the ever-present media.

It is vital and necessary for contact to be made between the medical team in charge during the Tour de France and the cyclists and their doctors, but some basic principles of medical practice need to be observed.

RECOMMENDATIONS

- *The cyclists' privacy should be respected.*
- *Examinations and, in particular, recording the cyclists' medical history should be carried out in an atmosphere of peace and quiet in order to obtain as much information as possible from the cyclists.*
- *The procedures for measuring weight and height should comply with normal technical procedures.*
- *It is vital that the cyclist's medical record is supplied and that the team doctor is interviewed.*
- *Closer cooperation with the UCI medical team and the issuing the results of the blood analyses carried out in Switzerland will definitely benefit this large medical structure put in place for the Tour de France.*

4. OUT-OF-COMPETITION TESTING BEFORE THE START

The day before the start, the UCI decided to control at random two cyclists who had "suspect" biological profiles.

On the morning of the prologue another cyclist was also tested. The three riders' urine samples were screened for erythropoietin.

The IO team was present at one of the tests carried out by a team made up of the Anti-doping Inspector and a doctor from the UCI. The test was carried out in accordance with the technical and administrative procedures set out by the UCI's Anti-doping regulations. The three samples were transported to the laboratory along with the samples taken after the prologue, which was 34 hours after the first out-of-competition sample was taken.

The observers did not see the conditions for storing and ensuring the security of these three samples taken during a random test between the time they were taken and their arrival at the venue for the anti-doping test during the prologue.

The observers agree with the UCI's strategy of testing at random cyclists with "suspect" results from the health checks.

RECOMMENDATIONS

The observers recommend that the samples are transported immediately to the laboratory after being taken and not 34 hours afterwards.

5. IN-COMPETITION TESTING

The French Ministry of Sport, the UCI and ASO shared responsibility for in-competition testing. The Ministry of Sport appointed a doctor responsible for all the samples taken.

The selection of the athletes took place every day one hour before the finish in the UCI Anti-doping Inspector's car in the presence and with the cooperation of the doctor appointed by the Ministry of Sport.

In accordance with UCI Anti-doping regulations, at each stage, the wearer of the yellow jersey and the stage winner were automatically selected to take an anti-doping test. During several stages the Anti-doping Inspector received a message from the President of the UCI Anti-doping Commission instructing him to select directly a few riders with "suspect" profiles from the blood tests.

Usually three other riders were then selected randomly to give a total of six or seven riders to be tested and two reserve riders. Half an hour before the finish, the anti-doping inspector would give the President of the board of commissioners the jersey numbers of the riders selected for testing, as well as those of the reserve riders. This information would then be passed on by the President via the Tour radio (accessible to Tour officials) twenty minutes before the finish. The names and numbers of the riders were then displayed at the entrance to the anti-doping control station.

During the time-trial stages lots would be drawn before the first rider set off and notice would be given by a UCI commissioner five minutes before each selected rider set off. After the finish the rider had an hour to report to the doping control station without ever being accompanied.

The Ministry of Sport doctor took the urine samples in two caravans made available by the ASO, one of which was used as a waiting room. The doping control station was at least 50 metres from the finish line and the press rooms. It was surrounded by barriers with a door opening onto the course. There was always a security guard from the organisation at the door.

The Berlinger ® system was used to take the samples and the forms from the Ministry of Sport were used as report templates.

Once all the tests were carried out, the doctor taking the samples and the Medical Inspector would put all the in-competition samples and the out-of-competition samples from the same day in a case containing dry ice required for transporting the samples at minus 20 degrees Centigrade.

The case was immediately brought to the heliport or airport where a helicopter or plane chartered by the organisation was waiting each evening to take the case and the samples to Bourget airport in Paris where they were handled by a private carrier. This carrier brought the samples between 0900 and 1000 the following day to the Châtenay-Malabry anti-doping laboratory.

The IO team observed the system for selecting the riders, the notification system, the procedures for taking the samples and the team also monitored the riders after the finish until they reported to the testing area during the prologue and the first four stages. The observers also monitored the case containing the samples from the doping control station right to the premises of the company assigned with their transportation during the day of the prologue. At the end of anti-doping testing after the third stage, the observers monitored how the case was brought to the heliport.

During the Tour, 132 urine samples were taken in-competition (6 samples for fifteen stages and 7 samples for 6 stages).

Most of the procedures followed during the in-competition tests complied with the UCI Anti-doping regulations and/or WADA International Standard for Testing. However, the observers identified a few discrepancies with regard either to the UCI Anti-doping regulations or the International Standard for Testing.

a. SELECTION PROCESS

When the riders were selected during the second stage, one of those selected by the President of the UCI Anti-doping Commission was not notified at the finish due to an error when noting the numbers of the riders selected. During the selection process at the third stage, the inspector had made a mistake noting the figures in the jersey number of the selected rider. Because of the confusion from the previous day when a rider was omitted, the latter was selected automatically for testing that day by the President of the UCI Anti-doping Commission.

b. NOTIFICATION

During the prologue the cyclists were notified five minutes before the start. This meant that the cyclists who were not notified then still had the opportunity to take a stimulant before the start of the race, as they were certain not to be tested (unless they won!). Of the six cyclists tested during the prologue only one reported for testing more than 60 minutes after notification, without any comment from the inspector. The Anti-doping Inspector informed some of the athletes and managers that they had 60 minutes to report for testing.

During the road-racing stages notification was given via the Tour-radio 20 minutes before the end of the race. This meant that the riders who were not selected again had an opportunity to take a fast-acting stimulant because they knew for certain they would not be tested (unless they won!).

c. ESCORTS

There were no escorts. The cyclists sometimes took over 20 minutes to get changed in their team trucks. Some kind of manipulation could have taken place.

d. TESTING AREA

There was no sign available to indicate where the anti-doping control station was. In addition, the testing area's location was not indicated in the route guide. The anti-doping caravan (waiting room and area where samples were taken) and the relevant doctor's car had a "Contrôle Médical" (Medical Test) sign rather than any mention of anti-doping.

In one instance, a cyclist and his doctor were meant to report for the anti-doping test, but could not find the venue due to the lack of signs.

The premises where the tests were carried out were far too small. There was also no system for recording when people came and left the area.

Every day there were unauthorised people in the anti-doping control and waiting areas, such as chauffeurs, mobile-home drivers and sometimes even members of the media.

With a UCI doctor's authorisation, a television crew was able to enter the restricted area and film the doping control station with only a WADA observer present. Afterwards, the TV crew remained in the restricted area around the caravan and filmed cyclists leaving the testing area, as well as the inside of the testing and waiting areas when a cyclist was there. Finally, the TV crew filmed a close-up of the procedure for filling and closing the dry ice container.

e. PROCEDURES FOR TAKING SAMPLES

Cyclists did not receive at any time an explanation of what the anti-doping test procedures involved.

Often the doctor taking the sample carried out himself the process for filling the samples without requesting the cyclist's authorisation or recording this action on the test form. Given the numerous (7) copies of the report which had to be made, the last copy, intended for the laboratory, was on several occasions virtually illegible.

The cyclist's privacy was not respected during micturition. This was carried out with the Anti-doping Inspector present, while the small bathroom was used to store the equipment and samples after they had been taken.

On at least one occasion, the doctor taking the sample left the caravan when the cyclist was trying to urinate. The Anti-doping Inspector was not able to observe the cyclist from where he was, although this was not part of his function.

The doctor taking the samples never measured their density and pH.

According to the doctor taking the samples, pH and density are not measured because of a directive from the LNDD (French National Drug Testing Laboratory) issued two years ago stating that this measure was not necessary.

The Independent Observers, however, questioned the Ministry of Sport on this matter, which informed them that neither UCI regulations nor French law made this measure compulsory, even if there was a relevant box for this purpose on the form and that it was stipulated by the Olympic Movement Anti-doping Code.

Whenever there was a sample containing an insufficient quantity of urine the doctor taking the samples never used the Berlinger® system intended for this purpose (with blue caps). A cyclist held his open sample in his hand, at one moment he was even left all alone in the doping control station. Despite this, the doping control form provides a section for indicating the number of the intermediate seals, which was therefore never used.

The doctor taking the samples always observed the 75 ml limit. Sometimes, when analysing EPO, a larger quantity of urine is required. To obtain this it would have been preferable to pour more urine into the bottles even if there was already a sufficient quantity of urine.

On several occasions the doctor took more than 75 ml urine, the quantity taken was noted in the report. He then filled bottle A and bottle B up to the label and the rest of the urine was poured down the toilet.

The doctor taking the samples noted all the drugs featuring in the rider's health booklet on the form and asked him to state all the drugs he had recently taken. He did not ask him to state the nutritional supplements he had taken. The doctor often did not ask the rider if he had any comments to make about the procedure.

After a cyclist was tested the samples were not kept in a safe, refrigerated place. The Berlinger® set with the sample was placed on the ground in the small bathroom in the caravan, which was not used for micturition.

During the prologue controls, the three samples from the random control taken in the morning were kept refrigerated in the carry case, which was not sealed and was located outside the caravan near the entrance.

In one case, the cyclist's copy of the doping control form was detached before the Cycling Director could sign the form. He signed it afterwards but the cyclist's copy did not have his signature on it. The various copies were put in envelopes and sent by normal post to the relevant authorities. The copies were therefore not kept in sealed envelopes.

Once the in-competition tests were complete, the container of dry ice was emptied in the street in full public view, with the samples placed on the ground nearby.

Then all the samples were placed in the container again and the driver of the doctors' car put the dry ice back in the container using plastic bags to protect his hands.

The actual container was not sealed. The unsealed envelope containing the copies of the laboratory reports and the sample security form were placed under the lid of the container, which did not close properly. This envelope was therefore in full public view and easily accessible.

f. TRANSPORT

There was no transport form (security form for the case).

After the prologue, the doctor taking the samples and the Anti-doping Inspector took the container to their hotel. Immediately when they arrived, the container was given to an employee from Dynaposte® (who was not requested for proof of identity). This company did not supply the doctor taking the samples with a transport form. This meant that the doctor had no proof that the samples had actually been delivered. Dynaposte® transported the samples to a post office where they were kept in air-conditioned premises. The post office's security system consisted of an alarm and access code. Dynaposte® does not have a quality control system. The samples were scheduled to be dispatched to the laboratory at 9 AM the following morning.

RECOMMENDATIONS

The following measures are recommended by the IO team as a means of improving the anti-doping test procedures at the Tour de France:

- The procedures for selecting the riders should be carried out in an atmosphere of peace and quiet to prevent any mistakes being made.*
- UCI Anti-doping regulations should describe precisely when the riders should be notified during the time-trial and road-racing stages. The form described in the UCI Anti-doping regulations should only be used for notification after the finish, preferably in the mixed area or alternatively, beside the relevant team trucks.
The Observers believe that this system can be totally practicable, even during a major competition like the Tour de France.*
- Once notification has been given, an escort trained specially for this purpose should accompany the rider until he arrives at the anti-doping control station, as described in the UCI Anti-doping regulations (Article 53) and in accordance with Article 5.4 of the International Standard for Testing.*
- The time the rider has to report for testing specified in the UCI Anti-doping regulations (Article 54 – 30 minutes or 50 minutes, if he has to attend a press conference) should be observed.*
- The testing area should be clearly signposted from the finish line (Article 38 of the UCI Anti-doping regulations).*

- *The testing area should comply with UCI recommendations (Article 39), especially with regard to its dimensions, guaranteeing riders' privacy during the test, as well as with Article 6.3 of the International Standard for Testing.*
- *The person appointed to guard the entrance to control station should have a system for recording who enters and leaves the area. The press, organisation drivers, mobile home drivers and other persons not involved in the anti-doping test process should not enter the testing area (Article 40 of the UCI Anti-doping regulations).*
- *The doctor taking the samples should explain the procedures to the riders, give them the opportunity to ask questions and take all the samples in a calm environment and in accordance with Article 47 of the UCI Anti-doping regulations and the procedures specified in WADA's International Standard for Testing (Article 7.0).*
- *The doctor taking the samples must ask riders which nutritional supplements they have taken, as this information could help with the interpretation of a positive analysis report and the decision on what kind of sanction to impose (Article 10.5 of WADA's World Anti-doping Code).*
- *The french doping control form should be amended so that there are fewer copies or that the copy to be sent to the Laboratory is the third or fourth sheet so that it is more legible. The IO team does not understand why one copy has to be supplied to the National Federation and a second to the International Federation.*
If it is an international competition the copy should be given to the International Federation and if it is national it should be given to the National Federation. The IO team wonders why a copy has to be supplied to the French Ministry of Sport if the French Council for the Prevention and Fight against Doping already receives one.
- *The doctor taking the samples should pour all the urine collected into bottles A and B because it is sometimes the case that the laboratory needs a large amount of urine to be able to confirm a quantifiable substance or detect erythropoietin.*
- *The samples should be kept refrigerated in a secure place after they have been taken.*

- *The doctor taking the samples and the UCI Anti-doping Inspector should place the copies of the report in the envelopes intended for the various recipients after the last sample has been taken and close them using the security seals (Article 66 of the UCI anti-doping regulations).*
- *The doctor taking the samples should draft a report after each doping control session. A single report at the end of the Tour mission would not allow for corrective measures to be taken if there were irregularities in one of the controls during the actual Tour.*
- *The samples and envelope containing the reports to be sent to the laboratory should be placed in a case, which should be sealed by the doctor taking the samples and the Anti-doping Inspector.*
- *The doctor taking the samples or the Anti-doping Inspector should complete a security document for the case (in accordance with Article 9.3.2. of the International Standard for Testing), specifying the date and time at which the case was sealed and the security seal number. The integrity of each person who will carry the case should be guaranteed and a new entry must be made in the security document when the case is received.*
- *The Tour's organisation should choose a company with a quality assurance system to transport the case.*
- *The samples should be transported in a case refrigerated at a temperature between 0°C and 10°C while in transit.*
Transporting the samples at -20°C is secure but it slows down the procedures for preparing the samples in the laboratory as they have to wait until the samples have thawed once they have arrived.

6. OUT-OF-COMPETITION TESTING DURING THE TOUR

A total of seven out-of-competition controls were carried out during the Tour de France (one on 7th July, two on the first rest day, one on 18th July and three on the second rest day). The doctor from the Ministry of Sport taking the samples carried out the controls in cooperation with the UCI Medical Inspector.

The President of the UCI Anti-doping Commission decided on the riders to be selected and the laboratory screened all the samples taken for erythropoietin.

One of the WADA observers observed one of the controls carried out at 7 AM on 7th July. It was carried out at the rider's hotel. The only remarks to be made relate on one hand to the time the rider took to report for the control – 23 minutes – after being notified by one of the team managers without being accompanied by the UCI Medical Inspector and on the other hand to the system for transporting the samples. After taking the samples, the doctor carried them in a small bag.

RECOMMENDATIONS

- *The UCI and French Ministry of Sport should carry out more controls, especially to monitor riders with "suspect" blood profiles, instead of monitoring them by selecting these riders for in-competition controls. Post-competition proteinuria can make it more difficult to interpret the results from the procedure for detecting erythropoietin in the laboratory.*
- *The UCI Medical Inspector should accompany the team manager from the time of their meeting until the rider is notified.*
- *The samples should be transported and stored in a refrigerated case, closed with a security seal until the time it is finally transported to the laboratory.*

7. LABORATORY

The analyses of all the samples taken for anti-doping testing, in competition and out of competition during the Tour de France, were carried out by the French National Drug Testing Laboratory (LNDD) in Châtenay-Malabry.

The LNDD is a national public, administrative institute, which operates under the responsibility of the Ministry of Sport.

This laboratory carries out analyses under the terms of Article L.3632-2 of the French Public Health Code and is responsible for managing and sending the equipment required to take the samples, as specified in the article of the Decree of 11th January 2001 mentioned above. Another of the LNDD's tasks is to carry out research in the area of doping prevention.

The laboratory has evolved historically as part of the institutional framework represented by national and international sporting bodies (sports federations, International Olympic Committee and World Anti-Doping Agency) and administrative bodies in the form of the Ministry of Sport and more recently (1999) the Council for the Prevention and Fight against Doping (CLPD).

In view of this, the laboratory's activities meet the requirements of national regulations and those of the sporting bodies.

At the moment, the LNDD is the only establishment in France approved by the IOC and WADA for carrying out analyses of anti-doping controls. In order to maintain the quality level of the service provided, the remuneration of the staff involved in carrying out the analyses does not depend on the number of samples processed nor on the results of these analyses. The LNDD is situated in the Châtenay-Malabry centre for popular education and sport (CREPS). It is made up of three technical departments, a paratechnical department, a quality assurance department, as well as a general secretariat. It has a current capacity for processing around 9,000 samples annually, based on 800 (between 700 and 900) per month over 11 months, taking into account a period of one month to deliver the results from the time the samples are received at the laboratory.

The Laboratory's management team is made up of the Director of the Laboratory, Prof. Jacques de Ceaurriz and a General Secretary. The Director is the laboratory's technical manager.

The LNDD has a staff of 40 comprising:

- 1 Director
- 1 General Secretary
- 3 Heads of department
- 1 Quality Assurance manager
- 23 technicians and 3 para-technical staff

- 8 administrators.

The laboratory has three technical departments. The various heads of department are also the technical managers for their respective departments:

- Department of GC chemical testing. This testing department is responsible for carrying out conventional analyses using gas chromatography, with or without mass spectrometry.
- Department for analytical research and development and LC chemical testing. Its function is to develop new analytical methods for identifying new doping products, as well as to improve already existing ones.
- Department for biological research and development and immunochemical testing. Its function is to develop new biological analytical methods for identifying new doping products, as well as to improve already existing ones. This department is responsible for the technical procedures of erythropoietin screening.

The laboratory's quality control system has had general accreditation from COFRAC (French Committee for Accreditation) in Medical Biology (no. 1-1174) since 1st June 2001.

The LNDD currently has the following equipment:

11 units – GC/MS

3 units – GC/MS-MS

2 units – (LC/MS)

2 units – system for analysing luminescence with dark room and optical system

1 unit – (LC/MS-MS)

An observer from the IO team visited the LNDD on the morning of 10th July. The observer was very warmly welcomed by the Director and quality assurance manager. He was able to visit the laboratory facilities, which are currently under renovation. These facilities are quite extensive offering an ideal separation between all the departments and sections. For instance, each technical department has a screening section, confirmation section and research section.

The observer witnessed the reception of the samples taken during the 4th stage, which arrived at the laboratory via Dynaposte® at around 10 AM.

The procedure for receiving the samples was carried out in a highly professional manner, in accordance with the laboratory's quality system and WADA's International Laboratory Standards. The samples were frozen at the time of their reception.

The observer was able to look at some of the laboratory's quality documents. The quality control system is well structured and implemented in a very active and highly professional manner.

The IO team concluded that the analysis reports described the methods used for screening and confirmation, but that these reports did not mention the technical procedure codes used.

The IO team also concluded that there were a few weaknesses concerning the security system. For example, a door leading outside remained open while the samples were being received; the WADA observer was not asked for identification at the entrance to the laboratory and his presence was not recorded.

The laboratory sign at the entrance on avenue Roger Salerno was not very visible, which made it difficult for any visitor trying to locate it.

During the Tour de France the Laboratory made small changes to the way in which it organised its daily activities. The samples from the Tour de France were processed as a priority and working hours were extended slightly, but the laboratory was not open at night or during the weekend.

Outside normal working hours and during the weekend, there was nobody at the laboratory, not even a security guard. The laboratory does, however, have a double security system:

- centrally controlled anti-burglary shutters on all the windows
- an alarm linked to a remote monitoring and response centre (at the Director or General Secretary's request).

While the WADA IO team was present at the Tour, following a telephone call between 3 and 4 PM, the laboratory sent the analysis reports by fax, ensuring anonymity was preserved, to the President of the IO team.

After the IO team's departure the Laboratory sent the analysis reports every day via the IO team President's confidential fax number in Lisbon.

The analysis reports were sent to the UCI Anti-doping Commission and the President of the French Council for the Prevention and Fight against Doping (CPLD) and for information, to the President of the International Olympic Committee's Medical Commission and to the person dealing with these matters at the French Cycling Federation.

During the Tour de France the LNDD processed a total of 142 samples (132 taken in-competition and 10 out-of-competition). In 2002, 138 samples were taken and 170 samples in 2001.

The in-competition samples taken during the prologue, 2nd, 4th, 6th, 7th, 8th, 9th, 12th, 13th, 14th, 15th, 16th, 18th and 19th stages and all the out-of-competition samples were screened for erythropoietin and hydroxyethyl starch (HES).

This type of screening was carried out on a total of 100 samples (70.4% of all the samples taken during the Tour), which marks an increase compared with the last few years (82 samples in 2002 and 72 in 2001).

All the samples were also screened for glucocorticosteroids.

The timetable for taking samples was drawn up by the French Ministry of Sport and the LNDD in cooperation with the UCI Anti-doping Commission. Either the Ministry or the UCI gave the laboratory the order to carry out the EPO analyses on the samples.

During the Tour de France the time taken for the results to be issued to the WADA IO team was on average around 66 and 72 hours after receipt of the samples at the laboratory for normal and EPO screening procedures respectively.

The time for issuing the results for normal procedures was around 100 hours for samples taken during the 12th and 14th stages. It must be pointed out that samples arrived at the laboratory every day, at least 14 hours after the in-competition tests had been completed.

Tables 1 and 2 show all the reports indicating the presence of doping agents.

Table 1

Reports indicating the presence of doping agents other than erythropoietin

| Substances | Number of samples | Comments on concentrations |
|-------------------------|--------------------------|--|
| Triamcinolone acetonide | 28 | Median – 6.0 ng / ml Variation – 1.0 ng / ml – 19 ng / ml |
| Betamethasone | 6 | Median – 26 ng / ml Variation – 1 ng / ml -37 ng / ml |
| Salbutamol | 6 | Median – 143 ng / ml Variation – 87 ng / ml -449 ng / ml |
| Dexamethasone | 3 | Median – 10 ng / ml Variation – 8 ng / ml -15 ng / ml |
| Caffeine | 1 | 10.7 µg / ml |
| Terbutaline | 1 | |
| Lidocaine | 1 | |
| Total: | 46 | |

Table 2

Reports indicating the presence of recombinant erythropoietin or with anomalies

| Classification | Number of samples | Comments |
|---|--------------------------|---|
| Presence of recombinant erythropoietin | 1 | |
| Undetectable recombinant erythropoietin | 15 | Camera intensity below 10,000 LAU |
| Unclassifiable recombinant erythropoietin | 4 | Electrophoretic migration between 48% and 65%, between 65% and 85% for NESP and for epoietin alfa and beta respectively |
| Total: | 20 | |

Looking at Table 2, it should be pointed out that 20% of the test results for the samples analysed for EPO had anomalies and that the only positive case was recorded in a sample taken out-of-competition.

The IO team has to emphasise the quality of the LNDD in terms of management, quality control system, facilities, staff and equipment. It would like to thank the laboratory's managers for their due cooperation throughout the whole of the Tour de France.

RECOMMENDATIONS

The IO team would like to make a few constructive recommendations with a view to optimize the services provided by the LNDD during the Tour de France:

- *The laboratory should reduce the time taken to issue results during the Tour de France. In a competition organised in stages like the Tour de France, a delay in announcing a positive result can allow a rider who has taken drugs to distort the competition results for a few days.*

To achieve this aim, the IO team recommends that the laboratory increase its working hours, by operating extra hours at night and during the weekend and that samples are transported at between 0°C and 10°C to facilitate the start of the technical procedures carried out in the laboratory.

- *The laboratory should have a system for receiving the samples 24 hours a day, which would facilitate the security system protecting the samples and the start of the technical procedures.*
- *The laboratory's security system should be reviewed to eliminate a few weak spots.*
- *The laboratory should enter in the report the codes for the technical procedures used in the analyses to make the information clearer for its customers.*

8. HANDLING THE RESULTS

The IO team would like to say at this point that, in addition to the analysis reports from the LNDD, it also received copies of the reports with the concealed identities of the riders from the doctor taking the samples, because the French law does not allow them to be disclosed.

The results were handled during the Tour de France by the UCI (UCI Anti-doping control regulations) and by the French Council for the Prevention and Fight against Doping (CPLD) (French Public Health Code – Article L 3612-1).

a. WHILE THE OBSERVERS WERE PRESENT

During this period the IO team received a copy of the reports either from the doctor taking the samples or the UCI Medical Inspector the day after each test, in competition or out of competition. The observers have nothing to report during this period.

b. AFTER THE OBSERVERS' DEPARTURE

During this period the President of the IO team received copies of the reports at his confidential fax number in Lisbon or by post.

On 25th July at 1440 (Paris time), the President of the IO team received a fax of analysis report no. 117/07-EPO, which confirmed the presence of recombinant erythropoietin in a sample taken on 18th July during an out-of-competition control. Twenty minutes later the President of the IO team received a telephone call from the President of the UCI Anti-doping Commission, who provided the same information and said that the UCI Medical Inspector was going to inform the rider and his cycling manager after the final stage of the day.

On 1st August the President of the IO team received the report on the B sample analysis carried out at UCI's request on 28th July, which confirmed the presence of recombinant erythropoietin.

On 4th August the President of the IO team requested additional information from the President of the UCI Anti-doping Commission concerning the disciplinary procedures applied in the event of a positive test, as well as information about the existence of other disciplinary procedures concerning positive results obtained by the LNDD, relating to samples taken during the Tour de France (copy of fax in Appendix IV).

On 6th and 7th August the President of the IO team received faxes (in Appendices V and VI) from the President of the UCI Anti-doping Commission informing him that the positive result case was handled in accordance with Articles 174 to 183 of the UCI Anti-doping regulations.

The UCI received the result of the B sample analysis after the final day of the Tour de France and for this reason, it was not possible to apply the principles described in Article 183 of the UCI Anti-doping regulations i.e. to exclude the rider from the race. The case was handed over to the rider's national federation, in accordance with Article 113 of the UCI Anti-doping regulations for it to apply disciplinary procedures, which must be completed within one month of the time limit set for the dispatch of the summons.

In a fax of 6th August the President of the Anti-doping Commission advised the observers that all the other positive cases were examined by the UCI Anti-doping commission, which decided that all these cases were justified on medical grounds. In the case of treatments taken during the Tour de France, all these treatments had been prescribed with the cooperation of the UCI's medical experts and were entered in the riders' health booklets.

The IO team reviewed the drugs declared in the reports and noted that in 71.8% of the samples taken the riders had declared that they had taken a drug. In 60.6% of the samples taken, glucocorticosteroids were administered and in 27.5% of cases beta-2-agonists were used.

The IO team confirmed that, in spite of the information from the UCI Anti-doping Commission about the existence of justified medical grounds in every case where there was a positive result for glucocorticosteroids, the timescales between the date the sample was taken and the date entered in the health booklet when the substance was administered were extremely large.

The IO team calculated these differences in twenty of the twenty-eight positive reports with triamcinolone acetonide, which was 37 days on average, with a variation between 8 and 57 days. In five cases the difference was more than 45 days. The IO team could find no reliable scientific data which could support a urinary excretion time of this duration.

On two occasions the IO team did not find that there was any medical justification concerning the cases showing a positive result for glucocorticosteroids:

- one positive result with triamcinolone acetonide with a medical declaration specifying Betamethasone by infiltration;
- one positive result with Betamethasone (30 ng/ml) with a medical declaration specifying cutaneous application of Betamethasone, the last occasion being 39 days before the test.

On the 18th August the President of the IO team requested additional information from the General Secretary of the CPLD concerning the disciplinary procedures applied with regard to the positive results received from the LNDD, relating to the samples taken during the Tour de France (copy of fax in Appendix VII).

On the same day the President of the IO team received a fax from the General Secretary of the CPLD (in Appendix VIII), informing him that, apart from the cases involving EPO, where proceedings were already under way, the President of the CPLD sent an initial letter to several riders to ensure that a "proof of a medical prescription based on justified therapeutic grounds" is sent to the CPLD.

RECOMMENDATIONS

The IO team recommends the following measures for improving the system for handling results in the Tour de France and other cycling competitions:

- *Results should be handled by an Anti-doping Commission with representatives from the UCI Anti-doping Commission and the CPLD to avoid any conflicts. A single code of regulations should be adopted, in accordance with WADA's World Anti-doping Code.*

- *The UCI should adapt its anti-doping regulations to allow temporary suspension after the result of the B sample analysis has been notified and in accordance with the guidelines applicable to temporary suspensions under the World Anti-doping Code (Article 7.5).*

Any rider who has obtained a positive result from the test carried out during the Tour de France may continue to participate in competitions until the final decision is made by his national federation, which has one or two months (if the national federation has a disciplinary appeal body) to complete the disciplinary procedures.

Until the final decision is made, the rider can help other riders in competitions organised in stages to achieve victories or a good position in the classification. Article 184a of the UCI Anti-doping regulations does not cover every case where temporary suspension proves to be necessary in order to ensure sporting equality.

- *It is necessary to carry out studies into the urinary excretion of glucocorticosteroids following administration of these products by inhalation, local injection, intra-articular injection or other forms of local application to verify the detection time for these substances in urine. We also recommend carrying out studies concerning the metabolism of cortisol in riders in order to detect a temporary or permanent inhibition of its production.*
- *The UCI Anti-doping Commission should be more careful in its analysis of substances and the dates entered in the health booklet when verifying the existence of justified medical grounds if, for instance, the Laboratory has detected a glucocorticosteroid, as specified in point 3 of Article 64 of the UCI's Anti-doping regulations.*

9. INFORMING AND EDUCATING ATHLETES

The day before the Tour de France started, the ASO organised a session to raise the riders' awareness with regards to doping issues in the reception room at the Paris City Hall. This session was attended by Patrice Clerc (President of the ASO), Jean-Marie Leblanc (Director of the Tour de France), Daniel Baal (Deputy Director of the Tour), Jean-François Pescheux (Competition Director), all the teams and their coaches and the members of the WADA IO team.

The organisers of the Tour, in particular, Jean-Marie Leblanc and Daniel Baal spoke in very clear and firm terms about observing the Tour de France's Code of Ethics and they reminded the riders that zero tolerance was applied in this competition.

Daniel Baal gave some explanations about the anti-doping controls which were to be carried out during the Tour and reminded the riders that the UCI would introduce for the first time during the Tour an analysis for free plasma haemoglobin with a view to detecting any possible use of synthetic haemoglobin. He announced that all the riders would receive the same leaflet as in 2002 entitled "Dopage et Cyclisme - ce que vous devez savoir" (Doping and Cycling - what you need to know), which contained very important and useful information for the riders (in Appendix IX).

Daniel Baal stressed the importance of the Tour de France's Code of Ethics (in Appendix X), which appears in the regulations and in the Tour guide book and was signed by all the teams.

This commitment was included both in the Agreement between the ASO and the International Association of Professional Cycling Groups (AIGCP) and in the Agreement signed with each team before the Tour started.

10. UCI MEDICAL COMMISSION

For the third time the UCI appointed a medical commission for the Tour comprising nine doctors. Based on a rota system, two of them were present at all times during the Tour. This commission's task was to observe, advise on and authorise the administration of drugs, especially those subject to restrictions.

The observers requested from the UCI to be informed of the Medical Commission's activities while they were present at the Tour. However, the observers never received any information about any requests made by any team doctor or rider to the Medical Commission.

The observers want to congratulate the UCI for setting up the Medical Commission.

11. MISCELLANEOUS

The French government has set up a system for importing drugs similar to the system introduced during the 2002 Tour.

This involves, in particular, recommendations about importing drugs, with an emphasis on the importance of professional team doctors having two medicine kits (an emergency kit containing possible doping substances and a backup kit without any doping substances).

It is also recommended that a list is kept up to date of all the drugs contained in the two kits being taken out and put back and that these documents can be shown, particularly during custom controls. The CPLD sent the ASO a document summarising these procedures with the reminder that doctors from foreign teams must declare the activities they intend to carry out when in France during the Tour de France. The observers congratulate the French government for this initiative.

The observers also have to congratulate the ASO for its reaction concerning the doping problematic and for implementing during the last few years measures capable of making a significant contribution to the fight against doping during the Tour de France. These include:

- Changes to the course so that fewer kilometres are covered in total and in the time-trial stages and easier stages with fewer hills.
- Ensuring that there are always two days' rest during the Tour with easier transfers.
- Producing a guide for hoteliers offering advice on the cyclists' food requirements and on measures for ensuring the riders get sufficient rest.

12. CONCLUSIONS

The Tour de France is one of the most important sporting events in the world, with huge media coverage and a considerable financial impact.

This is why the positive and negative aspects of this competition will attract so much media attention and therefore, may have major repercussions from an educational point of view.

The scandal during the 1998 Tour de France brought many changes in the fight against doping in almost every sport and in the world of cycling, in particular.

The changes made are moving in the right direction, based on closer cooperation between the responsible bodies (French Ministry of Sport, CPLD, UCI and the Tour de France's organisation) in developing a strategy to combat doping during the Tour de France. The meeting held before the Tour started, between the responsible bodies and WADA, is a good example to support the statement we have made. Accepting an Independent Observer team from WADA is another example of this positive development.

The anti-doping control system developed during the 2003 Tour de France involved considerable sums of money and sometimes was even excessively demanding (e.g. transporting the case containing the samples by aeroplane). It had weaknesses too, though, which may be highly significant in the pursuit of the ultimate goal – to protect the riders' health and retain the true spirit of sportsmanship, especially for the riders who do not use banned substances or methods.

The observers have no doubts at all about the good intentions of all those people involved in planning and implementing the system, but these small weaknesses may help possible cheaters to get round the system or find solutions they can use to defend their actions.

Procedures for taking samples might well be carried out under ideal conditions, but if there are riders who know for sure that they will not be tested twenty minutes before the finish line or even before they have started (time-trial stages) and have the opportunity to perform some kind of physical manipulation before they reach the doping control station, the system cannot guarantee sporting equality.

In spite of some weaknesses in the anti-doping control system, the observers would like to stress that there were strong, positive points to come out of the 2003 Tour de France:

- The firm tone adopted in the speech made by those responsible for organising the Tour de France about observing the Code of Ethics and the fight against doping.
- The soundness of the UCI's health check system and the important role this system plays in the fight against doping in cycling.
- The strategy developed by France in its fight against doping, especially in the area of legislation and through creating the CPLD, providing a high-quality anti-doping control laboratory and implementing measures to prevent trafficking of doping substances.

Closer cooperation between the bodies responsible for anti-doping controls during the Tour de France and the implementation of WADA's World Anti-doping Code and International Standards will be sufficient to guarantee an ideal system. However, all the measures implemented may not be enough if all the partners involved do not assume their responsibilities, especially with regard to protecting the riders' physiological limits. Furthermore, it is important to note that from now on, the observers will only carry out their mission if they have access to all the required documentation.

13. ACKNOWLEDGEMENTS

The observers would like to thank very sincerely all those who contributed to the success of this mission, in particular, the UCI, French Ministry of Sport, the ASO, CPLD and LNDD.

The President of the team would like to express his sincere gratitude to all the members of the IO team who were present during the 2003 Tour de France, where they demonstrated a high level of competence and availability.

14. MEMBERS OF THE INDEPENDENT OBSERVERS TEAM

- Prof. Dr. Luis Horta – Medical expert (POR)

President of the Independent Observers

Medical Doctor with a specialization in sports medicine, Director of the Lisbon Anti-doping Laboratory

- Dr. Anik Sax – Medical expert (LUX)

Independent Observer

Medical Doctor with a specialization in sports medicine, Department Head at the Institute of Sports Medicine in Luxembourg

- Ms. Jennifer Ebermann – Doping Control Expert (GER)

IO Programme Manager/Independent Observer

Manager, WADA

15. APPENDICES

Appendix 1: Procedure for determining blood parameters (UCI)



Juillet 03

Procédure de l'examen médical pour la détermination des paramètres sanguins

0. Conditions générales

Le laboratoire accrédité par l'UCI est l'Institut Indépendant qui assume la responsabilité opérationnelle scientifique et médicale globale de la récolte des échantillons sanguins et des paramètres biochimiques nécessaires à l'établissement de valeurs fiables de l'hématocrite.

Le responsable médical du programme est le Professeur Patrice Mangin.

Pour chaque examen l'UCI désigne **un ou plusieurs Inspecteurs Médicaux**.

La procédure se déroule dans un local adapté qui peut être une chambre d'hôtel. Si possible dans l'hôtel où **sont logés les coureurs, pour les prises de sang. L'appareil de mesure est installé dans un endroit pratique pour la remise des résultats.**

1. La prise de sang doit avoir lieu avant tout effort physique et de préférence avant le petit déjeuner.

A cette fin, l'Inspecteur Médical de l'UCI transmet personnellement le formulaire "Notification au coureur" au Directeur Sportif ou au Chef d'Equipe, à défaut au coureur (dans un délai raisonnable).

2. Le coureur désigné doit se présenter

- dans le délai mentionné sur la notification;
- au médecin de l'Institut Indépendant, dans la chambre mentionnée sur la notification.

Il est obligé de déposer sa licence auprès de l'Inspecteur Médical.

3. Le laboratoire désigné s'engage à assurer les prises de sang veineux chez les cyclistes qui lui sont désignés par l'UCI dans les règles de l'art et à effectuer les analyses dans les délais requis.

A cette fin, **le laboratoire** met à disposition l'équipe médicale scientifique et technique nécessaire; notamment pour la prise de sang, un médecin diplômé, familier des problèmes du sport. **Le laboratoire garantit l'expérience du médecin quant à l'exécution de prises de sang successives.**

Le matériel du prélèvement est fourni par **le laboratoire**.

Les tubes sont rendus anonymes au moment du prélèvement.

NB. Sur demande du coureur, la prise de sang peut également être pratiquée par le médecin du G.S., à la condition *sine qua non* qu'il se conforme au protocole établi. La

Procédure de l'examen médical pour la détermination des paramètres sanguins

présence de l'Inspecteur Médical est obligatoire. A défaut ou si l'échantillon de sang n'a pas été prélevé dès le 1^{er} essai, la ponction est effectuée par le médecin de l'Institut.

4. Après les prises de sang, l'équipe **du laboratoire** effectue les analyses requises.

Elles doivent répondre aux critères de qualité reconnus dans la profession.

A cette fin, l'on utilise un appareil « **COULTER PORTABLE DE LA SERIE A^c ● T ou SYSMEX XT-2000I** ».

Pour l'Hb synthétique, l'appareil « **HEMOCUE LOW PLASMA HEMOGLOBINE** » pourra être également employé.

Les résultats obtenus sur place seront communiqués immédiatement sous forme écrite au responsable désigné par l'UCI, soit l'Inspecteur Médical, soit un médecin de la Commission Sécurité et Conditions du Sport.

La totalité des paramètres sanguins sera communiquée au Président de la Commission Sécurité et Conditions du Sport.

5. L'Inspecteur Médical fait mention:

- A. des résultats aux Directeurs Sportifs ou Chefs d'Equipe concernés, en leur restituant la licence des coureurs examinés,
- B. des résultats inacceptables au Président du Collège des Commissaires au moyen du formulaire de l'UCI "Déclaration d'inaptitude",
- C. des valeurs hématocrite aux coureurs selon leurs désirs.

Cette procédure correspond aux conditions de travail idéales au déroulement des contrôles. Les déviations éventuelles ne peuvent donner lieu à des contestations, s'il n'est pas établi qu'elles ont pu influencer la validité des résultats.

Appendix 2: Measurement protocol (UCI)



Juillet 2003

Protocole de mesure de l'hémoglobine plasmatique libre

A. PRISE DE SANG – ECHANTILLONS A ET B

1. Le coureur doit être en position assise.
2. Le garrot ne doit pas être posé trop longtemps inutilement (délai de moins de une minute entre la pose du garrot et l'apparition du sang dans le tube).
3. Deux tubes de 2,7 ml de sang sont prélevés par une ponction unique. Ils sont désignés arbitrairement échantillons A et B. Ils sont étiquetés avec un numéro identique.
Le tube A est roulé au minimum 15 minutes et analysé immédiatement tel que la procédure le décrit ci-dessous dans le point C.
Le tube B est placé dans un flacon numéroté. Le flacon est attribué à l'équipe toute entière. Le numéro du flacon est inscrit sur le formulaire "Contrôle sanguin" sous le point 9.
Cet échantillon peut être utilisé pour une deuxième analyse, en cas de résultat entraînant une déclaration d'inaptitude.
4. Si pour une raison quelconque, le remplissage du deuxième tube présente des difficultés par la même ponction, il sera demandé au coureur s'il désire une deuxième ponction, sinon il admet qu'en cas de résultat entraînant une déclaration d'inaptitude pour l'échantillon A, la deuxième analyse se fera également sur l'échantillon A. Cette condition est acceptée sous le chiffre 11 du formulaire "Contrôle sanguin".
5. En cas de résultat entraînant une déclaration d'inaptitude pour l'échantillon A, le coureur peut demander que l'échantillon B soit ouvert devant lui et analysé suivant les mêmes règles que pour l'échantillon A. Après une contre-expertise, c'est le résultat de l'échantillon B qui sera pris en compte de manière définitive. La demande d'ouverture de l'échantillon B doit être formulée dans un temps raisonnable, après l'annonce du résultat de l'échantillon A, en tenant compte des impératifs de la course et de la qualité des analyses. Ce délai sera discuté le cas échéant entre l'inspecteur médical, le directeur sportif du coureur et le responsable scientifique de l'équipe médicale (cf. art 13.1.055 du règlement UCI).

C. ANALYSE

1. Préparation des sangs et mesures

Les sangs sont roulés pendant 15 minutes au minimum avant l'analyse pour homogénéisation et stabilisation de température.

Après la détermination des valeurs hématiques habituelles (Hct, Hb, réticulocytes), les échantillons seront centrifugés.

2. Coloration du plasma

Protocole de mesure de l'hémoglobine plasmatique libre

La couleur du plasma sera observée. Si le plasma a une **coloration rose/rouge**, l'échantillon sera alors analysé pour l'hémoglobine plasmatique libre, avec les appareils prévus (Coulter, Sysmex ou Hemocue).

Si la valeur d'hémoglobine plasmatique libre est supérieure à **300mg/dl (3g/l)**, on procédera à l'analyse de l'échantillon B.

- **Analyse de l'échantillon B**
 - Le coureur ou son mandataire est alors informé qu'il peut assister, dans un délai défini par l'inspecteur médical, à l'analyse de l'échantillon B.
 - La contre-expertise sera effectuée à l'aide de l'analyseur Coulter ou Sysmex ou Hemocue.
 - **Déroulement de la contre-expertise :**
 - **effectuer deux mesures à l'aide de l'analyseur Coulter ou Sysmex ou Hemocue**
 - **si la valeur est > à 300mg/dl (3g/l) le coureur est déclaré inapte.**

Appendix 3: Information for riders (UCI)



UNION CYCLISTE INTERNATIONALE
COMMISSION SECURITE ET CONDITIONS DU SPORT

Messieurs les coureurs,

Nous aimerions vous donner quelques informations concernant les contrôles sanguins.

Contrôles sanguins

Lors des contrôles sanguins, notamment pendant le Tour de France, les paramètres habituels seront analysés : hématocrite, hémoglobine et réticulocytes.

Si les analyses devaient montrer des profils anormaux (valeurs ou évolutions anormales), on demandera au coureur (ou à son médecin) quelle est l'origine de cette anomalie, et on lui fera savoir qu'il sera davantage contrôlé, car il restera suspect à nos yeux.

Dès cette année, nous utilisons un nouveau protocole pour identifier les coureurs qui ont un profil sanguin non normal.

Il est évident qu'on les soumettra à des contrôles antidopage, pour la recherche de l'EPO ou du NESP.

Hémoglobines synthétiques

Depuis le début de l'année, nous mesurons, lors des contrôles sanguins, un nouveau paramètre : **l'hémoglobine plasmatique libre**. Ce paramètre augmente de façon très importante (plusieurs centaines de fois) en cas d'administration d'hémoglobine synthétique. Comme vous savez, l'Hb synthétique est une substance interdite, et sa commercialisation à plus large échelle est en train de se réaliser. Or, vu qu'elle est encore indétectable lors des contrôles urinaires, il nous fallait trouver une solution pour décourager son éventuelle utilisation, afin de garantir le droit à avoir des compétitions équitables.

Ce paramètre ne peut pas être employé en tant que test antidopage et, par analogie à ce qui se passe avec les valeurs d'hématocrite élevées, un coureur qui aura une valeur d'**hémoglobine plasmatique libre > 300mg/dl (3g/l)** sera donc **déclaré inapte**, et obligé à se soumettre aux investigations hématologiques. L'hémoglobine synthétique, grâce à la détermination de ce paramètre, peut être détectable pendant plusieurs jours.

Nos équipes médicales vont analyser ce paramètre lors de tous les **contrôles sanguins du matin (y compris celui d'aujourd'hui)**, qui se dérouleront de la même façon qu'auparavant (même quantité de sang prélevée).

En restant à votre disposition pour tout renseignement supplémentaire, nous vous souhaitons tout le succès sportif que vous méritez.

Dr Leon Schattenberg
Président Commission Antidopage

Dr Mario Zorzoli
Médecin UCI

CH 1860 Aigle / Suisse
☎ +41 24 468 58 11 fax +41 24 468 58 12
www.uci.ch

Appendix 4: Request for information 04/08/03

LABORATÓRIO DE ANÁLISES E DOPAGEM
Av.ª. Prof. Egas Moniz (Estádio Universitário)
1600 LISBOA - PORTUGAL

TELEFAX Nº.: 21 797 75 29

De: Prof. Doutor Luís Horta
Para: President of the Medical
Commission of UCI
FAX Nº.: 00 31 46 400 85 21

Nº. de Páginas: 1

Data: 04/08/2003

Dear Dr. Leon Schatewberg

As President of the Independent Observer Mission during the Tour de France, I want kindly request information about the development of the disciplinary procedures done in the positive case met in the sample A 190775 and confirmed in the sample B 190775, on 1st of August.

I want also to request if UCI develop any other disciplinary procedures concerning the positive reports delivery by the French Antidoping Laboratory in the samples collected during the Tour de France.

Best regards.



**President of Independent
Observer Mission**

Appendix 5: UCI reply (07/08/03)

7.AUT.2003 16:17

N9535

P.1



INTERNATIONAL CYCLING UNION

CH 1860 Aigle / Switzerland

☎ : +41 24 468 58 11 – Fax : +41 24 468 58 12

FAX MESSAGE

To : Prof Dr. Luis Horta
Fax nbr : +351 21 797 75 29
From : Dr. Leon Schattenberg
Date : 7 August 2003
Ref : Antidoping Services / Lsch / cv
Total pages : 1 (including this one)
Subject : ***Independent Observer Mission***

Dear Dr. Horta,

I would like to add some information to my last correspondence, As regard to the duration of the proceedings and according art. 113 AER: *"The proceedings before the competent body of the licence-holder's national federation must be completed within one month of the time limit set for the dispatch of the summons."*

We will of course keep you informed all along the procedure. All the documents we will get from the national federation shall be sent to you.

Best Regards,

On behalf of the Antidoping Commission,

A handwritten signature in black ink, appearing to be 'L. Schattenberg', written over a horizontal line.

Dr. Leon SCHATTENBERG, President

Appendix 6: UCI reply (06/08/03)

6 AUG 2003 14:49

N2485

P.1



INTERNATIONAL CYCLING UNION
CH 1860 Aigle / Switzerland
☎ : +41 24 468 58 11 – Fax : +41 24 468 58 12

FAX MESSAGE

To : Prof Dr. Luis Horta
Fax nbr : +351 21 797 75 29
From : Dr. Leon Schattenberg
Date : 6 August 2003
Ref : Antidoping Services / Lsch / cv
Total pages : 2 (including this one)
Subject : **Independent Observer Mission**

Dear Dr. Horta,

I would like to give you the following information as regard to your demand concerning the Tour de France 2003.

The positive case found in the sample A+B 190775 has been and is being managed according to the UCI AER, art. 174 to 183 (Stage races).

The UCI was informed by the laboratory of the B sample result after the Tour de France ended. Therefore, the case has now been transmitted to the rider's national federation for disciplinary procedure.

As far as the corticoids are concerned, the results have been examined by the UCI Antidoping Commission. All cases were justified on medical grounds and were accepted by the Antidoping Commission. For treatments prescribed during the Tour de France, all of them have been prescribed with the cooperation of the UCI medical experts and treatments have been written in the health booklet.

I also invite you to read two articles written by Dr. Gérard Guillaume:

1. Corticothérapie locale et effet systémique (revue de la littérature) publié dans Médecin du Sport ; Gérard Guillaume et Marcel-Francis Kahn ; e-mail address : g-quillaume@wanadoo.fr
2. Intérêts et limite des infiltrations de corticoïdes dans le sport, publié dans journal de traumatologie du sport.

I will try to send you other information and publication as soon as possible.

Best Regards,

On behalf of the Antidoping Commission,



Dr. Leon SCHATTENBERG, President

Appendix 7: Fax to the CPLD (18/08/03)

Presidência do Conselho de Ministros
Secretaria de Estado da Juventude e Desportos


Instituto do Desporto de Portugal
Laboratório de Análises e Dopagem

FAX

| | | | |
|-----------------------------------|---|----------------------------|----------------------|
| PARA: (To) | CPLD MR. PHILIPPE ROUX-COMELI SÉCRÉTAIRE-GENERALE | REFERÊNCIA: (REFERENCE) | 049/L.A.D./2003 |
| A/A: | | DATA: (DATE) | 18 DE AGOSTO DE 2003 |
| DE: (FROM) | INSTITUTO DO DESPORTO DE PORTUGAL – LABORATÓRIO DE ANÁLISES E DOPAGEM | FAX: | 00 33 1 4062 77 39 |
| N.º PAGINAS: (NUMBER OF PAGES) | 1 | | |
| ASSUNTO: | TOUR DE FRANCE | | |

Cher Philippe Roux-Comeli,

Comme President de la Mission d' Observateurs Independents de l' AMA dans le Tour de France, j'ai reçu les copies des PV sans l' identification des coureurs et les rapports analytiques du LNDD.

J' ai reçu plusieurs rapports analytiques positives pour glucocorticosteroids. J'ai demande des informations à l' UCI sur la gestion de ces resultats. L' UCI a informé que sa Comission Médicale a décidé que tous les rapports positives avec glucocorticosteroids étions justifié par une declaration médicale dans le carnet de santé.

Je suis entrain de finir le rapport de notre mission et je veux savoir si le CPLD a quelque chose a ajouter a la position de l' UCI.

Salutations.



Luis Horta
President de la Mission O. I. de l' AMA

AV: PROF. EDUAR MONTE (ESTÁDIO UNIVERSITÁRIO) – 1600-190 LISBOA
TEL: (351)21 798 50 73 – FAX: (351)21 797 75 28

E-MAIL: cmd.labor@mail.telepac.pt

Appendix 8: Reply from the CPLD (18/08/03)

| | |
|--|---|
| <p>CONSEIL DE PRÉVENTION ET DE LUTTE CONTRE LE DOPAGE</p> <p>— ♦ —</p> <p>39, rue Saint Dominique 75007 PARIS</p> <p>----</p> <p><i>Le Secrétaire Général</i></p> | <p><i>République Française</i></p> <p>Paris, le 18/08/2003</p> <p>— ♦ —</p> <p>Tél : 01.40.62.76.76 Fax : 01.47.53.75.36</p> <p><u>Caractère</u> : très urgent <input checked="" type="checkbox"/> urgent <input type="checkbox"/> courant <input type="checkbox"/></p> |
|--|---|

EXPEDITEUR : Philippe ROUX COMOLI

**DESTINATAIRE : Dr Luis HORTA, Président de la mission O.I. de
l'AMA**

N° DE FAX : 00.35.1.217977529

Monsieur le Président,

Le Président du Conseil de prévention et de lutte contre le dopage a reçu l'ensemble des procès-verbaux et résultats d'analyse relatifs aux contrôles effectués lors du Tour de France 2003.

Outre le cas portant sur l'EPO dont la procédure est en cours, il a transmis un premier courrier à plusieurs coureurs afin de s'assurer que ceux-ci transmettent au Conseil « la preuve d'une prescription médicale à des fins thérapeutiques justifiées ». L'instruction est donc en cours.

En restant à votre entière disposition pour toute information complémentaire, je vous prie d'agréer, Monsieur le Président, l'expression de mes sentiments les meilleurs.


Philippe ROUX COMOLI

Nombre de pages y compris celle-ci : 1



Introduction

Chaque sportif souhaite améliorer ses performances.

La compétition étant au cœur du sport, surtout lorsqu'il se pratique à un haut niveau, il est légitime de chercher à se dépasser. Toujours plus haut, toujours plus fort !

Pour y parvenir, certains sont prêts à toutes les compromissions, y compris à tricher. Certains sont prêts à tous les sacrifices, y compris à se détruire la santé. Le dopage est un miroir aux alouettes. Il donne au corps l'illusion d'accroître ses capacités. En fait, sous dopage, le corps va artificiellement au-delà de ses limites. Cela n'est pas sans conséquences.

Ce guide vise à vous informer sur les risques liés au dopage pour votre santé. Il a pour objectif de vous montrer que le dopage est loin d'être anodin.

Ce guide est également destiné à vous montrer à quel point l'idée du dopage est éloignée du sport. Faire du sport, c'est d'abord prendre soin de son corps. Ceci est vrai quel que soit le niveau auquel on le pratique.

Parce que vous êtes des professionnels, ce message vous concerne tout particulièrement. Vous pratiquez un sport de très haut niveau et la tentation du dopage peut être très présente. Il vous concerne aussi parce que vous êtes un exemple pour les jeunes. Il importe de leur montrer, en particulier à ceux qui courent dans quelques années sur les routes du Tour de France, que l'on peut être performant sans se dopage. Car se dopage, c'est mettre en péril sa santé : pour vous-même, pour les autres sportifs et pour le sport, refuser le dopage c'est vital !

Qu'est-ce que le dopage ?

Pour un sportif, se dopage consiste à consommer des produits interdits ou à recourir à des méthodes prohibées. Dans tous les cas, l'objectif est d'obtenir une amélioration artificielle de ses performances physiques.

Il existe un grand nombre de produits dopants. Schématiquement, on peut les classer en deux grandes catégories : les médicaments et les stupéfiants. Parmi les médicaments, on trouve notamment les bêta-bloquants, les corticoïdes, les anabolisants, les anesthésiques locaux, les diurétiques et l'hormone de croissance.

Un grand nombre de stupéfiants peut être utilisé comme produit dopant. Citons en particulier les stimulants comme la caféine, comme le cannabis, la méthadone, l'héroïne et la morphine, figurent également parmi les stupéfiants.

Tous ces produits ne sont pas totalement interdits. Certains d'entre eux sont à usage restreint. C'est-à-dire qu'ils ne peuvent être utilisés que dans certaines conditions. C'est par exemple le cas des anesthésiques locaux, des corticostéroïdes et des bêta-bloquants.

Par ailleurs, certaines techniques sont utilisées dans le cadre du dopage. Ces méthodes interdites sont le dopage sanguin et les manipulations chimiques, pharmacologique ou physique visant à masquer les substances dopantes dans les échantillons d'urine utilisés lors des contrôles anti-dopage.

Vrai ou Faux ?

Les produits dopants sont dangereux par eux-mêmes.

Faux

C'est la façon de les consommer qui est dangereuse. Certains des produits utilisés pour se dopage sont des médicaments très utiles pour soigner des maladies. Ainsi, il faut bien distinguer l'usage de ces produits et l'abus de leur consommation. C'est l'abus qui est dangereux et qui conduit bien souvent à la dépendance.

Quels sont les risques du dopage ?

Le dopage fait principalement peser des risques pour la santé. La consommation excessive de produits dopants entraîne ainsi de nombreux effets nocifs. Ces derniers peuvent survenir rapidement ou, à l'inverse, apparaître longtemps après la prise du produit dopant (jusqu'à plusieurs années après). A chaque produit sont associés des effets nocifs.

Citons quelques exemples :

Les stimulants (caféine, amphétamines...)

Ils peuvent entraîner des troubles cardio-vasculaires (troubles du rythme cardiaque, infarctus du myocarde, accident vasculaire cérébral), des troubles neurologiques et neuro-musculaires (troubles de la coordination, par exemple) et des troubles psychiques (agressivité ou dépression entre autres).

Les stéroïdes anabolisants

Chez l'homme, ils provoquent des atteintes au niveau de la prostate et du foie, une augmentation de la taille des seins, des troubles du fonctionnement des testicules, une production réduite des spermatozoïdes (avec un risque d'infertilité).

Les diurétiques

Ils peuvent provoquer une déshydratation aiguë, des troubles cardio-vasculaires, une fatigue et un état de faiblesse généralisée, ainsi qu'un dysfonctionnement des reins.

Les hormones peptidiques

(EPO, hormones de croissance, DHEA) : les principaux effets indésirables de ces produits sont la survenue d'une insuffisance cardiaque sévère (pouvant entraîner un infarctus du myocarde, voire une mort subite), d'un diabète, de troubles sexuels et de cancers.

Tous les sportifs recourant à des produits dopants ne présentent pas systématiquement l'ensemble de ces troubles. Il existe en fait une variation importante d'une personne à une autre. Certains seront plus sensibles que d'autres aux effets indésirables d'une consommation de substances dopantes, sans que l'on puisse prédire à l'avance le degré de sensibilité de chacun. Mais il n'existe pas d'individu qui soit totalement insensible aux effets néfastes des produits dopants.

Le saviez-vous ? Le saviez-vous ?

On considère que la durée de vie des sportifs qui se dopent est inférieure d'une vingtaine d'années en moyenne à celle de la population générale

Comment se passer du dopage ?

Le dopage n'est pas une fatalité, même dans le sport de très haut niveau. De très nombreux champions ont connu de grande carrière sans recourir aux produits dopants. Comment ont-ils fait, comment vous pouvez faire ?

En fait, pour pratiquer un sport, quel que soit son niveau, sans user de substance dopante, il n'y a pas de « recette miracle » ! La performance repose sur les principes de base que sont l'entraînement, la récupération, l'hygiène de vie, le suivi médical et le travail d'équipe.



5 conseils pour améliorer vos performances sans dopage

1 L'entraînement : c'est la base de toute pratique sportive. Sans entraînement, on ne peut maintenir son organisme en situation de réaliser l'activité que l'on souhaite pratiquer. On ne peut pas non plus progresser dans ses performances. L'entraînement doit être adapté à chaque personne, en modulant l'intensité, le volume et la répétition des activités physiques.

2 La récupération : elle est tout aussi essentielle. Sinon, l'organisme se fatigue à l'excès et il devient moins performant. La récupération repose sur l'alternance « travail - repos » qui doit respecter le rythme entre l'éveil et le sommeil. Les techniques de relaxation, le stretching (méthode basée sur des étirements) et l'hydratation favorisent la récupération.

3 L'hygiène de vie : pratiquer un sport suppose une hygiène de vie adaptée. Celle-ci nécessite de respecter ses rythmes biologiques. Par ailleurs, chaque sportif sait combien l'alimentation est importante. Il est donc essentiel d'adopter de bonnes habitudes alimentaires.

4 Le suivi médical : il est très important d'être suivi sur le plan médical très régulièrement. Le médecin doit pouvoir ainsi s'assurer de vos aptitudes à votre activité sportive, tout à la fois sur le plan médical, biologique et psychologique. La relation engagée avec le médecin doit permettre de mettre sur pied une véritable « stratégie de santé ».

5 Le travail d'équipe : dans le cyclisme, la performance est le fruit du travail de toute une équipe. Vous le savez, auprès de vous intervient l'entraîneur, le préparateur physique, le kinésithérapeute, le psychologue et le médecin. Il est important que chacun exerce ses talents dans le respect des autres et des règles. C'est comme cela que les équipes gagnent !

Photos : J.-C. Moreau - B. Bode - ASO



Dopage et cyclisme ce que vous devez savoir

PACTE ÉDITIONS BENZUS - VIGNON DE SAINT-MANVILLE
100 rue de la République - 91000 Evry-Courcouronnes

Appendix 10: Tour de France Code of Ethics



>> Le code éthique du Tour de France

Le Tour de France possède en propre des valeurs sans lesquelles il perdrait non seulement sa crédibilité sportive, mais encore son indiscutable fonction culturelle, économique et sociale.

Ces valeurs de référence conservent une signification à travers le temps, tout en prenant en compte les notions de compétition, d'affrontement, de dépassement et en finalité l'objectif majeur de victoire.

Mais il ne peut s'agir ni de n'importe quelle compétition, ni de victoire obtenue à n'importe quel prix.

Comme toutes les activités sportives, comme les autres épreuves cyclistes et davantage encore parce qu'il est la plus prestigieuse de toutes, le Tour de France doit donc s'accompagner :

- De règles précises, dont ni la lettre ni l'esprit ne doivent être transgressés.
- Du respect des officiels chargés de l'application de ces règles et des décisions qu'ils peuvent être amenés à prendre.
- De l'égalité des chances offertes aux concurrents. L'avantage accordé à l'un d'entre eux d'une manière illicite est contraire à l'éthique sportive. C'est en ce sens que le dopage, trop souvent présent dans l'activité sociale ordinaire, est inadmissible en sport.
- D'une action rigoureuse menée contre la tricherie, la corruption et toute forme d'arrangement permettant de vaincre autrement que par les moyens du sport.

L'acceptation de ces principes conditionne l'estime et la popularité qu'accorde au Tour de France un public nourri par une longue période d'exploits et de légende.

Les champions d'aujourd'hui ont en héritage un patrimoine qui ne saurait aller sans les vertus morales qui ont contribué à le forger.

Tout coureur cycliste, quels que soient la place qu'il occupe et le niveau de ses performances, est par conséquent tenu de respecter cette éthique fondamentale. A défaut, il risquerait de conduire son sport vers la perversion et la décadence.





GDC00734

CURRICULUM VITAE

Bruce A. Goldberger, Ph.D., DABFT

Professional

Address:

Forensic Toxicology Laboratory
Diagnostic Reference Laboratories
Department of Pathology, Immunology and Laboratory Medicine
College of Medicine
University of Florida
4800 S.W. 35th Drive
Gainesville, Florida 32608

| | |
|----------|--|
| phone | office – (352) 265-0680 ext. 72001 |
| | laboratory – (352) 265-0680 ext. 72002 |
| | cellular – (352) 494-7569 Nextel DC – 160*19775*1 |
| fax | (352) 265-9904 |
| pager | (888) 443-2911 |
| e-mail | bruce-goldberger@ufl.edu |
| homepage | www.pathology.ufl.edu/~bgoldber/ |

EDUCATION

May, 1982

B.A. degree in Zoology
Drew University
Madison, New Jersey

August, 1985

M.S. degree in Forensic Toxicology
University of Maryland School of Medicine
Department of Pathology, Division of Forensic Pathology
Baltimore, Maryland

Thesis: “*In vitro* and *in vivo* studies of the collection and delayed analysis of the alcohol content in breath.”

January, 1993

Ph.D. degree in Forensic Toxicology
University of Maryland School of Medicine
Department of Pathology, Division of Forensic Pathology
Baltimore, Maryland

Dissertation: “Measurement and interpretation of heroin, 6-acetylmorphine and morphine concentrations in biological tissues obtained from heroin users and heroin-related deaths.”

PROFESSIONAL POSITIONS

| | |
|---------------------------------------|--|
| July, 2005 to present | Professor, Clinical Track Department of Pathology, Immunology and Laboratory Medicine Department of Psychiatry University of Florida College of Medicine Gainesville, Florida |
| November, 2002 to June 2005 | Associate Professor, Clinical Track Department of Psychiatry University of Florida College of Medicine Gainesville, Florida |
| July, 2001 to June 2005 | Associate Professor and Director of Toxicology, Clinical Track Department of Pathology, Immunology and Laboratory Medicine University of Florida College of Medicine Gainesville, Florida |
| July, 1999 to June, 2001 | Assistant Professor and Director of Toxicology, Clinical Track Department of Pathology, Immunology and Laboratory Medicine University of Florida College of Medicine Gainesville, Florida |
| October, 1994 to June, 1999 | Assistant Professor and Director of Toxicology Department of Pathology, Immunology and Laboratory Medicine University of Florida College of Medicine Gainesville, Florida |
| September, 1987 to September, 1994 | Toxicologist National Center for Forensic Science a division of Maryland Medical Laboratory Baltimore, Maryland |
| September, 1989 to May, 1992 | Assistant Toxicologist and Toxicology Laboratory Manager Toxicology Laboratory Office of the Chief Medical Examiner Baltimore, Maryland |
| July, 1986 to September, 1989 | Assistant Toxicologist Toxicology Laboratory Office of the Chief Medical Examiner Baltimore, Maryland |

September, 1982
to September, 1987

Laboratory Technologist
Clinical Toxicology Department
Maryland Medical Laboratory
Baltimore, Maryland

CERTIFICATIONS

Diplomate. American Board of Forensic Toxicology, Certificate Number 218 (3/1/99-6/30/09)
Clinical Laboratory Director. Board of Clinical Lab Personnel, Department of Business and Professional Regulation, State of Florida, License Number DI 0033647 (5/18/95-8/31/06)
Forensic Toxicology Specialist. American Board of Forensic Toxicology, Certificate Number 5001 (11/1/92-2/28/99)
Toxicological Chemist. National Registry of Certified Chemists (previously known as the National Registry in Clinical Chemistry), Certificate Number 2254 (1991-2006)

HONORS AND AWARDS

Educational Research Award. Society of Forensic Toxicologists, 1984 and 1986
Sunshine Award. Toxicology Section, American Academy of Forensic Sciences, 1988
Toxicology Section Scholarship. American Academy of Forensic Sciences, 1991
Outstanding Scientific Achievements by a Young Investigator Award. American Association for Clinical Chemistry, 1994
Mid-Career Achievement Award, The International Association of Forensic Toxicologists, 2004
Alexander O. Gettler Award, Toxicology Section, American Academy of Forensic Sciences, 2006

CAMPUS ACTIVITIES

Teaching

College of Medicine:

General Pathology and Immunology (BMS 5608) – Lectures in Chemical Carcinogenesis and Forensic Pathology
Systemic Pathology and Laboratory Medicine (BMS 5600) Case Study Mentor
Principles of Drug Action (GMS 6002) – Forensic Pharmacology (Grand Rounds)
Special Topics in Pathology: Cellular & Molecular Basis of Liver Disease (GMS 6381) – Lecture in Pharmacogenomics and Drug Toxicity
Translational Neuroscience: Junior Honors Medical Program (MEL 4001) – Forensic Medicine
Laboratory Rotation Mentor, Interdisciplinary Graduate Program
Pathology Resident Rotation (Preceptor)

College of Pharmacy:

Selected Topics in Pharmacy (The Role of the Pharmacist in Substance Abuse Education and Prevention; PHA 4933) – Lecture in Forensic Toxicology

College of Law:

Handling Drug/Alcohol Crimes Seminar – Lecture in Forensic Toxicology

Thesis/Doctoral Dissertation Committee Member:

Michael W. Belford, Department of Chemistry, 2001-2003
 Matthew W. Warren, Department of Psychiatry, 2003-2006
 Timothy L. Naylor, Department of Physiological Sciences, 2003-present
 Frank Kero, Department of Chemistry, 2004-present
 David Khey, Department of Criminology, Law and Society, 2005-present

Student/Fellowship Training:

| <u>Name</u> | <u>Program</u> | <u>Date</u> |
|------------------------------------|---|--------------|
| Diana Garside | Post-Doctoral Fellowship in Forensic Toxicology | 1994-1997 |
| Jeri Roper-Miller | Graduate Student – Doctoral | 1994-1998 |
| Ruth Winecker | Graduate Student – Doctoral | 1994-1996 |
| Jason Byrd | Graduate Student Rotation | 1996 |
| Bart Wacek | Graduate Student Intern | 1996 |
| Mary Rucker | Graduate Student – IDP Lab Rotation | 1998 |
| Richard Fox | Center for Precollegiate Education and Training | 1998 |
| Gretchen Miller | Graduate Student – Master's | 1998-1999 |
| Beth Ladlie | Graduate Student – IDP Lab Rotation | 1998 |
| Tara Sabo | Graduate Student – IDP Lab Rotation | 1999 |
| Andria Hobbs | Undergraduate Student – University Scholars Program | 1999-2000 |
| Kristofer Rau | Graduate Student – IDP Lab Rotation | 1999 |
| Jessica Walrath | Graduate Student – IDP Lab Rotation | 2000 |
| Bruno De Martinis | Visiting Scientist (Brazil) | 2000-2001 |
| Karen Vieira | Graduate Student – IDP Lab Rotation | 2001 |
| Michele Merves | Graduate Student – Doctoral | 2001-2007 |
| Sheng-Meng Wang | Visiting Scientist (Taiwan) | 2001 |
| María Antonia Martínez González | Visiting Scientist (Spain) | 2002 |
| Michele Merves | Graduate Student – Doctoral | 2002-present |
| Rebecca Murray | Pharmacy Student | 2002-2003 |
| Ansley Gascoigne | Undergraduate Intern (UCF) | 2004 |
| Kelly MacDougall | Graduate Student – IDP Lab Rotation | 2005 |
| Rebecca Fidler | Undergraduate Intern (UCF) | 2005 |
| Jennifer Hoyer | Undergraduate Intern (UCF) | 2005 |

| | | |
|---------------------|--|------|
| Christopher Moody | Undergraduate Intern (UCF) | 2005 |
| Melissa Clarady | Undergraduate Intern (UCF) | 2006 |
| Kimberly Fitzgerald | Medical Student (UF College of Medicine) | 2006 |
| Melanie Atkinson | High School Student (PK Young) | 2006 |
| Jenna Chin | Undergraduate (UF Senior Research) | 2006 |
| Sarah Guilmain | Graduate Student – IDP Lab Rotation | 2006 |

Administration

College of Medicine

Member, Medical Selection Committee, 2005-present

Department of Pathology, Immunology and Laboratory Medicine:

Director, William R. Maples Center for Forensic Medicine
Unit Director of Toxicology, Diagnostic Referral Laboratories
Member, Diagnostic Referral Laboratories Executive Committee
Member, Diagnostic Referral Laboratories Quality Assurance Group
Chairman, Biosafety Committee
Biologic Safety Officer
Member, Clinical Research Committee
Member, Rocky Points Labs, Building & Safety Committee

Interdisciplinary Toxicology Graduate Training Program (Center for Environmental and Human Toxicology):

Faculty Member
Graduate Coordinator for Pathology
Member, Toxicology Graduate Specialization Committee

University of Florida University Athletic Association:

Member, University Athletic Association Substance Abuse Committee

OTHER ACADEMIC ACTIVITIES

Adjunct Assistant Professor in Clinical Chemistry. University of Maryland School of Medicine,
Department of Medical and Research Technology, Baltimore, Maryland, 1991-1992 and
1993-1994

Thesis Advisor. College of Graduate Studies, Thomas Jefferson University, Philadelphia,
Pennsylvania, 1994

External Examiner for Sarah Kerrigan. Faculty of Graduate Studies, The University of British
Columbia, Vancouver, B.C. Canada, 1997

PROFESSIONAL MEMBERSHIPS AND ACTIVITIES

American Academy of Forensic Sciences (AAFS), Fellow, 1983-present

Academy Activities

Program Committee, Poster Session Chairman, 1994
Nominating Committee, 1996-1997
Council, 1995-1997
Program Committee, Breakfast Seminars Chairman, 1997-1998
Program Committee, Poster Session Chairman, 1998-1999
Strategic Planning Committee, 1997-1998
Long Term Planning Committee, 1998-present
Board of Directors (Toxicology Section Representative), 1999-2002
Academy-Wide Luncheons, Moderator, 2000
Program Committee, Workshop Chairman, 2000-2001
Forensic Sciences Foundation Nominating Committee, 2000-2001
Program Committee, Program Co-Chairman, 2001-2002
Program Committee, Program Chairman, 2002-2003
Policy and Procedure Committee, 2002-present
AAFS Website Redesign Task Force (Chairman), 2002-2003
AAFS Website Content Oversight Task Force, 2003-2004
Vice President, 2003-2004
Membership Committee, Chairman, 2003-2004
Treasurer, 2004-2006
Executive Committee, 2004-present
Finance Committee, 2006-present
Forensic Sciences Foundation Nominating Committee, 2005-2006
President-Elect, 2006-2007
Trustee, Forensic Sciences Foundation, 2006-2008
President, 2007-2008

Toxicology Section Activities

Workshop Co-Chairman, 1992-1995, 1999
Program Committee, 1992-1996
Program Chairman, 1994-1995
Secretary, 1995-1996
Chairman, 1996-1997
Chairman, Nominating Committee, 1997-1998
Abstract Guidelines Committee, 1995-1996
Editor, *News and Views*, 1993-1996
Steering Committee, 1994-present
Minutes Review Committee, 1994-1999
Awards Committee, 1997-1999, 2001-present
ad hoc Membership Guidelines Committee, 1994-1999

American Association for Clinical Chemistry (AACC), Member, 1984-present

TDM/Tox LIP Committee, 1991-1996; Chairman, 1995-1996
FUDT/LIP Committee, 1997-present; Chairman, 1999
Toxicology News Committee, Chairman, 2000-2001

Workshop Leader, 1993-1994
TDM and Clinical Toxicology Division, representative to the Joint Committee on Education and Training in Toxicology, 1993-1996
American Board of Forensic Toxicology (ABFT)
Board of Directors, 2000-present
Treasurer, 2002-present
Promotion Committee, 1998-present
Examination Committee, 1999-present
Finance Committee, 2002-present
California Association of Toxicologists (CAT), Associate Member, 1993-present
Council of Science Editors, 2001-present
National Academy of Clinical Biochemistry, Fellow, 2003-present
International Association for Chemical Testing (IACT), Member, 1999-present
National Committee for Clinical Laboratory Standards, Member, 1993-2002
Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs Committee
National Registry of Certified Chemists (NRCC)
Board of Directors, 2001-2003
Examination Committee, 2001-2003
National Safety Council, Committee on Alcohol and Other Drugs, Member, 1988-present
Education and Training, Co-Chairman, 1990-1991
Education and Training, Chairman, 1992-1997
Executive Board, 1992-present
Action Programs Subcommittee, 1998-present
Web-Site (ad hoc) Subcommittee, Chairman, 2002-present
Society of Forensic Toxicologists (SOFT), Member, 1984-present
Advisory Committee on Hair Analysis, 1991-1992
Annual Meeting Program Committee, 1993-present
Chairman, SOFT Web-site Committee, 1997-present
Chairman, SOFT Logo Redesign Committee, 1997
Board of Directors, 1998-2000
Special Issue Editor, Journal of Analytical Toxicology, 1999
Board of Directors (ex officio), 2005-present
The International Association of Forensic Toxicologists (TIAFT), Member, 1991-present
T'2007 Scientific Advisory Committee, 2006-present

EDITORIAL TASKS

Clinica Chim Acta – Member, Editorial Board, 1999-2003
Clinical Chemistry - ad hoc reviewer, 2006
Clinical and Forensic Toxicology News (AACC Newsletter) – Chairman, Editorial Advisory Board, 1999-2001
Drug Court Review – ad hoc reviewer, 2005
Forensic Science International – ad hoc reviewer, 2004-2006
Forensic Science Review – Member, Board of Editors, 2002-present
Journal of Analytical Toxicology –
Editor-in-Chief, 2001-present

Member, Editorial Advisory Board, 1997-2000
Invited Reviewer, 1995-1996
Society of Forensic Toxicologists Special Issue Editor, October, 1999
Journal of Forensic Sciences – Member, Editorial Board, 1997-present
Science & Justice – ad hoc reviewer, 2005
Therapeutic Drug Monitoring and Toxicology (AACC In-Service Training and Continuing Education Publication) – Chairman, Editorial Board, 1995-1996
Clarke's Isolation and Identification of Drugs (3rd Edition), Pharmaceutical Press – Member, Editorial Board, 2000-present

CONSULTANT AND OTHER RELATED ACTIVITIES

Expert Witness/Consultant in Forensic Toxicology. Qualified as an Expert in Forensic Toxicology in Federal, State of Florida, Canadian, and Military Courts of Law
Laboratory Inspector. National Laboratory Certification Program, Substance Abuse and Mental Health Services Administration, DHHS, 1989-2000
Chairman. Florida Department of Law Enforcement/Institute of Police Technology and Management Implied Consent Rules Committee, 1999-2000
Chairman. Substance Abuse Committee, Athletic Programs, Santa Fe Community College, 1997-present
Co-Chair, Methadone Associated Mortality: A National Assessment Workshop, Center for Substance Abuse Treatment Substance Abuse and Mental Health Services Administration, Arlington, Virginia, May 2003
Member. Florida Task Force on Suicide Prevention, Office of Drug Control, Office of the Governor, State of Florida, 2003-present
Consultant, Drug Enforcement Administration (Arlington, VA), 2004-present
Consultant, National Football League Players Association (Washington, D.C.), 2006-present

REVIEW OF GRANTS AND CONTRACTS

Consultant. Counterdrug Technology Assessment Center Demand Reduction Advisory Board, Office of National Drug Control Policy, Executive Office of the President, 1993
Consultant. Maternal Lifestyle Study, National Institute of Child Health and Human Development, National Institutes of Health, 1992-1993
Consultant. Measurement of Caffeine, Paraxanthine and Osmolality in Serum from the Collaborative Perinatal Project, National Institute of Child Health and Human Development, National Institutes of Health, 1996
ad hoc Committee Member. Molecular, Cellular and Chemical Neurobiology Research Review Subcommittee [NIDA/B], National Institute on Drug Abuse Initial Review Group, National Institutes of Health, 1996-1997
ad hoc Reviewer. Clinical Protocols, Addiction Research Center, National Institute on Drug Abuse, National Institutes of Health, 1998
ad hoc Committee Member. Epidemiology and Prevention Review Committee [NIDA/G], National Institute on Drug Abuse Initial Review Group, National Institutes of Health, 1998
ad hoc Reviewer. NIDA/INVEST Research Fellowship Program, National Institute on Drug Abuse, National Institutes of Health, 2000

Reviewer. FY 2005 General Forensics Research and Development Solicitation, Office of Science and Technology, National Institute of Justice, U.S. Department of Justice, 2005

Reviewer. FY 2006 Forensic Toxicology Research and Development (R&D) Solicitation, Office of Science and Technology, National Institute of Justice, U.S. Department of Justice, 2006

INVITED LECTURES

The Use of Quality Control by Forensic Urine Drug Testing Laboratories. Hewlett-Packard Clinical/Forensic Seminar, Rockville, Maryland, April, 1989.

Principles of Forensic Toxicology. Forensic Toxicology Workshop, Department of Health and Rehabilitation Services, State of Florida, Orlando, Florida, April, 1990.

Drug Testing in the 1990s. 1991 Hewlett-Packard User's Group Meeting, American Society of Mass Spectrometry, Nashville, Tennessee, May, 1991.

Optimization of GC/MS. Hewlett-Packard User's Group Meeting, Baltimore, Maryland, October, 1991.

FPDT: Forensic Pelage Drug Testing (Breakfast Seminar). American Academy of Forensic Sciences, New Orleans, Louisiana, February, 1992.

Optimization of GC/MS. Forensic and Clinical Drug Analysis by GC/MS. Hewlett-Packard Sponsored Workshop, American Association for Clinical Chemistry, Chicago, Illinois, July, 1992.

Confirmation. Preparing for the NIDA NLCP Inspections: Problems Commonly Encountered by Inspectors. Society of Forensic Toxicologists, Cromwell, Connecticut, October, 1992.

Hair Testing: The Growing Way to Test for Drugs of Abuse. The Pittsburgh Conference, Atlanta, Georgia, March, 1993.

Disposition of Heroin and 6-Acetylmorphine in Hair. Clinical Pharmacology/Toxicology Forum, Baltimore, Maryland, March, 1993.

Testing for Abused Drugs in Human Hair. 1993 Hewlett-Packard User's Group Meeting, American Society of Mass Spectrometry, San Francisco, California, May, 1993.

Hair. Drug Analysis of Unusual Biological Tissues. American Association for Clinical Chemistry, New York, New York, July, 1993.

Heroin and Metabolites in Biological Tissues Obtained from Heroin Users and Heroin-Related Deaths. Roche Diagnostics Seminar, Branchburg, New Jersey, August, 1993.

Seminar: Measurement and Interpretation of Heroin, 6-Acetylmorphine and Morphine Concentrations in Biological Tissues. Department of Medical and Research Technology, University of Maryland School of Medicine, Baltimore, Maryland, September, 1993.

Study of the Effect of "UrinAid" on HHS Regulated Urine Specimens. Drug Testing Advisory Board, Division of Workplace Programs, Substance Abuse and Mental Health Services Administration, Bethesda, Maryland, September, 1993.

Current Status of Hair Drug Testing. Capital Section, American Association for Clinical Chemistry, Baltimore, Maryland, November, 1993.

Optimization Techniques for GC/MS. The In's and Out's of Capillary Gas Chromatography: Routinely Utilized Inlet and Detector Systems. American Academy of Forensic Sciences, San Antonio, Texas, February, 1994.

Opiates. Forensic Toxicology. Armed Forces Institute of Pathology, Vienna, Virginia, April, 1994.

Adulteration Issues-Laboratory Checks and Balances. Substance Abuse Workshop. Department of Energy, Alexandria, Virginia, May, 1994.

- Hair. Drug Analysis of Unusual Biological Tissues. American Association for Clinical Chemistry, New Orleans, Louisiana, July, 1994.
- Pharmacology of Heroin and Related Opiates in Hair. SOFT Conference on Drug Testing in Hair, Tampa, Florida, October, 1994.
- Opiates. Fundamentals of Forensic Toxicology: A Basic Course. Society of Forensic Toxicologists, Baltimore, Maryland, October, 1995.
- Testing for Drugs in Hair. Seminar. Departments of Pharmacodynamics and Pharmaceutics, University of Florida College of Pharmacy, Gainesville, Florida, October, 1995.
- Postmortem Toxicology. Advances in Alcohol and Drug Testing-Significance for Legal Proceedings. Toxicology Consultants Forensic Science CLE Seminar Series. Fort Lauderdale, Florida, December, 1995.
- The Analysis of Anabolic Agents in Sports. Amateur Athletic Drug Testing – Substance Abuse Policy. American Academy of Forensic Sciences, Nashville, Tennessee, February, 1996.
- Drugs of Abuse Testing. Boehringer Mannheim Corporation 1996 Preferred Partners Meeting and Product Fair. Orlando, Florida, March, 1996.
- Drug Free Workplace Seminar - Lab Procedures. Gainesville Area Chamber of Commerce, Gainesville, Florida, May, 1996.
- Testing for Cocaine in Hair: New Clinical and Forensic Applications. Department of Pathology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, Florida, June, 1996.
- Drug Testing for Drug Courts: Components of Reliability. Gaining Momentum: Drug Courts in Florida. Supreme Court of the State of Florida, Office of the State Courts Administrator, Naples, Florida, June, 1996.
- Rohypnol and Rape Prevention. Rape & Crime Victim Advocate Program, Alachua County Department of Community Services, Gainesville, Florida, July, 1996.
- Medical Examiner Toxicology, Analysis of Unusual Biological Tissues, and Forensic Chemistry. Introduction to Forensic Toxicology. American Association for Clinical Chemistry, Chicago, Illinois, July, 1996.
- High Sensitivity CEDIA Benzodiazepine DAU: New Solutions for the Nineties. Boehringer Mannheim Corporation 1996 Industry Workshop at the AACC Annual Meeting. Chicago, Illinois, July, 1996.
- Forensic Toxicology. 1996 Clinical Chemistry and Toxicology Audioconference Series - Teleconference Network of Texas. Gainesville, Florida, August, 1996.
- Forensic Toxicology Sites on the World Wide Web. Forensic Toxicology and the Internet. Society of Forensic Toxicologists, Denver, Colorado, October, 1996.
- Drug Free Workplace Seminar – Lab Procedures. Gainesville Area Chamber of Commerce, Gainesville, Florida, October, 1996.
- Forensic Science: A Living Science – Amniotic Fluid and Breast Milk. American Academy of Forensic Sciences, New York City, New York, February, 1997.
- Forensic Toxicology: QA/QC Considerations. NFSTC Forensic Toxicology Workshop, Gainesville, Florida, August, 1997.
- Implementation of a PT Program for Hair Testing in Florida. HHS Drug Testing Advisory Board, Scientific Meeting on Drug Testing of Alternative Specimens and Technologies, Arlington, Virginia, September, 1997.

- Signs of Impairment & Physiology and Pharmacology, Breath Test Instructor Course, Institute of Police Technology and Management, University of North Florida, Jacksonville, Florida, January, 1998.
- Cocaine Analyses in Breast Milk and Nails. Seminar. Department of Pharmaceutics and Pharmacodynamics, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois, March, 1998.
- Hewlett-Packard Seminar on Drug Testing and Forensic Analyses. Wilmington, Rockville, Atlanta, and Tampa, March, 1998.
- Results from the First Round of the State of Florida Hair Proficiency Testing Program. Drug Testing Advisory Board, Division of Workplace Programs, Substance Abuse and Mental Health Services Administration, Bethesda, Maryland, June, 1999.
- Physical Effects of Abuse. The GHB, GBL and 1,4 Butanediol Working Group Meeting. Sponsored by Orphan Medical Group, National Forensic Science Technology Center, St. Petersburg, Florida, July, 1999.
- Case Studies in Forensic Toxicology. Department of Pathology, Immunology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, Florida, August, 1999.
- Forensic Toxicology. Microbiology and Cell Science Student Organization. University of Florida, Gainesville, Florida, September, 1999.
- Hewlett-Packard Seminar on Drug Testing. Fundamental and Critical Procedures Used in the Analysis of Drugs of Abuse in Urine by GC/MS. Mexico City, Mexico, November, 1999.
- Leading Edge Seminar – The Future of Forensic Medicine at the University of Florida, University of Florida Department of Conferences and Seminars, Division of Continuing Education, Gainesville, Florida, February, 2000.
- Forensic Toxicology of Opiate Alkaloids and Synthetic Analgesics – Metabolism of Opioids. American Academy of Forensic Sciences, Reno, Nevada, February, 2000.
- Post-Mortem Forensic Toxicology (Part 1), Shands Hospital Laboratories Continuing Education Program, Gainesville, Florida, March, 2000.
- Drug Testing: An Important Element in Substance Abuse Prevention – Today's Drugs, Beating the Test and Other Myths. American Alliance for Health, Physical Education, Recreation and Dance, Orlando, Florida, March, 2000.
- Contemporary Practice in Clinical Toxicology – Opiates. American Association for Clinical Chemistry, Alexandria, Virginia, June, 2000.
- Assessment of Alternative Specimens in Forensic Toxicology. Society of Forensic Toxicologists, Milwaukee, Wisconsin, October, 2000.
- Post-Mortem Forensic Toxicology (Part 2), Shands Hospital Laboratories Continuing Education Program, Gainesville, Florida, November, 2000.
- The Drunk and Drugged Driver: Understanding Toxicology, Advanced DUI Seminar, Florida Prosecuting Attorneys Association, Ocala, Florida, March, 2001.
- Effects on the Body, Florida Statewide Conference on Designer Drugs, Florida Department of Law Enforcement, Orlando, Florida, April, 2001.
- Club Drugs, Florida Association of Medical Examiners, Daytona Beach, Florida, November, 2001.
- Forensic Toxicology, Florida Homicide Investigators Association, Gainesville, Florida, November, 2001.
- Chemical Agents, Community Bioterrorism Summit, Shands HealthCare, Gainesville, Florida, November, 2001.

- Analytical Toxicology Considerations in Drugs and Driving Cases, The Effects of Drugs on Human Performance and Behavior, Robert F. Borkenstein Center for Studies of Law in Action, Indiana University, Bloomington, Indiana, March, 2002.
- Chemical Agents of Terrorists, The Challenge of Bioterrorism, Florida League for Nursing, University of Florida, Gainesville, Florida, April, 2002.
- Chemical Agents, Shands Hospital Laboratories Continuing Education Program, Gainesville, Florida, August, 2002.
- Club Drugs. Microbiology and Cellular Science Student Organization. University of Florida, Gainesville, Florida, September, 2002.
- Analytical Toxicology Considerations in Drugs and Driving Cases, The Effects of Drugs on Human Performance and Behavior, Robert F. Borkenstein Center for Studies of Law in Action, Indiana University, Bloomington, Indiana, March, 2003.
- Drug and Deaths in Florida, Grand Rounds, Department of Psychiatry, University of Florida College of Medicine, March 2003.
- Forensic Pharmacology, Consumer Chemistry (CHM 1083), University of Florida, April 2003.
- The Role of the Toxicology Laboratory in the Prosecution of Drugged Driving Cases, International Association for Chemical Testing, Cocoa Beach, Florida, April, 2003.
- The Challenge to the Forensic Community. Methadone Associated Mortality: A National Assessment Workshop, Center for Substance Abuse Treatment Substance Abuse and Mental Health Services Administration, Arlington, Virginia, May, 2003.
- Methadone Deaths in Florida. Methadone Associated Mortality: A National Assessment Workshop, Center for Substance Abuse Treatment Substance Abuse and Mental Health Services Administration, Arlington, Virginia, May, 2003.
- Drug Testing Update, Department of Community Health and Family Medicine. University of Florida College of Medicine, Gainesville, Florida, September, 2003.
- Forensic Toxicology of Methadone – Methadone and Death Investigations. Society of Forensic Toxicologists, Portland, Oregon, October, 2003.
- Methadone Overdose Deaths. National Association of Drug Diversion Investigators, Fort Lauderdale, Florida, November, 2003.
- Pursuing a Career in the Forensic Sciences. Biomedical Research Career Development Seminar, University of Florida Interdisciplinary Program in Biomedical Sciences, Gainesville, Florida, January, 2004.
- Methadone-Associated Mortality: Report of a National Assessment – Toxicological Issues, The 6th International Conference on Pain & Chemical Dependency, Brooklyn, New York, February, 2004.
- Ephedrine: Drug or Supplement? Ephedrine Related Compounds and the Debate on Their Potential for Contribution to Injury – Analytical Issues of Ephedrine and Related Compounds in Possible Injury Cases. American Academy of Forensic Sciences, Dallas, Texas, February, 2004.
- Developing Global Strategies for Identifying, Prosecuting, and Treating Drug-Impaired Drivers – Chemical Sensing of Exhaled Breath, Tampa, Florida, February, 2004.
- Ninth Annual Southern Coastal International Conference – Breath, Blood and Urine Testing, Jekyll Island, Georgia, March, 2004.
- Club Drugs, Shands Hospital Laboratories Continuing Education Program, Gainesville, Florida, April, 2004.

- Analytical Toxicology Considerations in Drugs and Driving Cases, The Effects of Drugs on Human Performance and Behavior, Robert F. Borkenstein Center for Studies of Law in Action, Indiana University, Bloomington, Indiana, September, 2004.
- Club Drugs, Shands Hospital Laboratories Continuing Education Program, Gainesville, Florida, December, 2004.
- Forensic Toxicology Reference Laboratory and Public Service, Southeastern Association of Pathology Chairs and Department Administrators, St. Petersburg, Florida, January, 2005.
- Evidenced Based Forensic Science: Interpreting Postmortem Toxicology in the Light of Pathologic Findings – Interpreting Postmortem Opioid Measurements. American Academy of Forensic Sciences, New Orleans, Louisiana, February, 2005.
- Drugs and Drug Deaths in Florida. University of Florida Committee on Alcohol and Other Drug Education and Policy, Gainesville, Florida, April, 2005.
- OTC and Prescription Stimulants, Stimulants Workshop (sponsored by Society of Forensic Toxicologists), Orlando, Florida, April 2005.
- Pharmaceutical Abuse, Annual Training for Demand Reduction Program Coordinators, Drug Enforcement Administration, Orlando, Florida, August, 2005.
- Update in Forensic Toxicology: Selected Topics in Death Investigation – Case Studies in Analytical & Forensic Toxicology, North American Congress of Clinical Toxicology 2005, American Academy of Clinical Toxicology, September, 2005.
- An Epidemic of Drug Deaths in Florida, Department of Pathology, Immunology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, Florida, September, 2005.
- Forensic Toxicology Update – Opiates. Society of Forensic Toxicologists, Nashville, Tennessee, October, 2005.
- Interpretation of Toxicological Analysis in the Elderly – Opioids in the Elderly. American Academy of Forensic Sciences, American Academy of Forensic Sciences, Seattle, Washington, February, 2006.
- Pediatric Postmortem Toxicology Session – Toxicological Findings of a Mother and Fetus in a Fatal DUI. American Academy of Forensic Sciences, American Academy of Forensic Sciences, Seattle, Washington, February, 2006.
- Annual Conference on Addictions – An Epidemic of Opioid-Related Deaths in Florida, Florida Society of Addiction Medicine, Lake Mary, FL, March, 2006.
- Methamphetamine: A Deadly Formula – Methamphetamine: Toxicology, Pathology and Treatment, Second Annual Prevention Summit, DISC Village, Inc., Tallahassee, FL, March, 2006
- Analytical Toxicology for Impaired Driving Programs – Laboratory Aspects, The Effects of Drugs on Human Performance and Behavior, Robert F. Borkenstein Center for Studies of Law in Action, Indiana University, Bloomington, Indiana, April, 2006.
- Forensic Toxicology, Ninth Forensic Science Educational Conference (FSEC) (sponsored by the American Academy of Forensic Sciences and Court TV), Florida Gulf Coast University, Fort Myers, Florida, May, 2006.
- CE Committee: How Does Your QA/QC Program Measure Up? – Analytical Toxicology – QA/QC Laboratory Aspects Society of Forensic Toxicologists, Austin, Texas, October, 2006.
- Alcohol Use Disorders Colloquium – Alcohol and Drugs in Trauma Cases, Shands at the University of Florida and the University of Florida, College of Medicine, Division of Acute Care Surgery, Gainesville, FL , February, 2007.

DUI and DUI Drugs: State of the Art, Florida Society of Addiction Medicine, Gainesville, FL, March, 2007.

STATE AND NATIONAL MEDIA APPEARANCES

In Search of Jesse James, A&E and History Channel, 1996
Party Drug, Fatal Drug, 48 Hours, CBS News, 2001
PMA, Channel One News, 2001
Addicted: An OxyContin Tragedy, 48 Hours, CBS News, 2002
Ecstasy – Nothing to Rave About, Florida's News Channel, 2002
Hooked On Club Drugs, VH1, 2002
State of Florida v. Rachel Sercey Trial, Court TV, 2003
State of Florida v. Laura Roberts, Inside Edition, 2004
Secrets from the Grave, 48 Hours, CBS News, 2005
Caffeine in Decaf Coffee, Today Show, 2006
Caffeine in Energy Drinks, National Public Radio, 2007
Death of Daniel Smith, CNN and MSNBC, 2007
Death of Anna Nicole Smith, Fox News and Court TV, 2007

PUBLICATIONS

Books

1. Liu RH and Goldberger BA (eds). Handbook of Workplace Drug Testing. AACC Press, 1995 (380 pages) [second printing 1996 – 396 pages; Chinese translation 1997 – 448 pages].
2. Jenkins AJ and Goldberger BA (eds). On-Site Drug Testing. Humana Press, Inc., 2002 (276 pages).

Chapters

1. Goldberger BA and Cone EJ: Heroin. *in* Encyclopedia of Analytical Science. Academic Press, 1995 (pp. 3861-3866).
2. Inoue T, Seta S and Goldberger BA: Analysis of drugs in unconventional samples. *in* Handbook of Workplace Drug Testing. AACC Press, 1995 (pp. 131-158).
3. Garside D and Goldberger BA: Determination of cocaine and opioids in hair. *in* Drug Testing in Hair. CRC Press, Inc., 1996 (pp. 151-180).
4. Winecker RE and Goldberger BA: Urine specimen suitability for drug testing. *in* Drug Abuse Handbook. CRC Press, Inc., 1998 (pp. 764-772).
5. Goldberger BA and Jenkins AJ: Drug Toxicology. *in* Sourcebook on Substance Abuse: Etiology, Epidemiology, Assessment, and Treatment. Allyn & Bacon, 1999 (pp. 184-196).

6. Kerrigan S and Goldberger BA: Opioids. *in* Principles of Forensic Toxicology. AACC Press, 1999 (pp. 202-220). {revised – 2003}
7. Magura S, Laudet A and Goldberger BA: Improving the validity of behavioral drug abuse research through drug testing. *in* Drug Testing Technology: Assessment of Field Applications. CRC Press, 1999 (pp. 215-233).
8. Garside D and Goldberger BA: Forensic and Medicolegal Issues. *in* Atlas of Hair and Nails. Churchill Livingstone, 1999 (pp. 227-232).
9. Kerrigan S and Goldberger BA: Drugs of Abuse - Body Fluids. *in* Encyclopedia of Forensic Sciences. Academic Press, 2000 (pp. 616-626).
10. Ropero JD and Goldberger BA. Opioids. *in* The Clinical Toxicology Laboratory - Contemporary Practice in Clinical Toxicology. AACC Press, 2001 (pp. 73-96).
11. Caplan YH and Goldberger BA: Blood, Urine and Other Fluid and Tissue Specimens for Alcohol Analyses. *in* Medical-Legal Aspect of Alcohol, fourth edition. Lawyers & Judges Publishing Company, 2003 (pp. 149-159).
12. Merves ML and Goldberger BA: Heroin. *in* Encyclopedia of Analytical Science, second edition. Elsevier Press, 2005 (pp. 260-266).
13. Kerrigan S and Goldberger BA: Forensic Toxicology. *in* Forensic Nursing. Elsevier Mosby Publishing, 2005 (pp. 123-139).
14. Kerrigan S and Goldberger BA: Substance Misuse – Alternative Body Fluids Analysis. *in* Encyclopedia of Forensic and Legal Medicine. Elsevier Press, 2005 (pp. 192-201).
15. Isenschmid DS and Goldberger BA: Workplace Testing – Analytical Considerations and Approaches for Drugs. *in* Drug Abuse Handbook, second edition. CRC Press, Inc., 2007 (pp. 775-799).
16. Merves ML and Goldberger BA: Quality Assurance, Quality Control, and Method Validation in Chromatographic Applications. *in* Chromatographic Methods in Clinical Chemistry and Toxicology. John Wiley & Sons, Ltd., 2007 (pp. 1-14).
17. Merves ML and Goldberger BA: Forensic Toxicology. *in* Forensic Chemistry. John Wiley & Sons, Inc. (in press).
18. Kerrigan S and Goldberger BA: Specimens of Maternal Origin – Amniotic Fluid & Breast Milk. *in* Drug Testing in Alternative Biological Specimens. Humana Press, Inc. (in press).

Monographs

1. Goldberger BA: Acetaminophen, Caffeine, Chloramphenicol, Lithium and Procainamide Drug Monographs. *in* Drug Monitoring Data Pocket Guide II. AACC Press, 1994.
2. Goldberger BA: Opiates. Abused Drugs Monograph Series. Abbott Diagnostics, 1994.
3. Goldberger BA, Roper-Miller JD, Zawta B and Jackson R: Drugs of abuse testing. Questions and Answers. Boehringer Mannheim Corporation, 1998. {revised and reprinted by Roche Diagnostics in 1999}
4. Hammett-Stabler C, Goldberger BA and Roper-Miller JD: Abused drugs. *in* Medical Toxicology Self Study. American Association for Clinical Chemistry, 1998.
5. Roper JD, Garside D and Goldberger BA. Opiates. *in* Contemporary Practice in Clinical Toxicology. American Association for Clinical Chemistry, 1998. {second edition, 2000}
6. Goldberger BA (ed.): Hair Analysis: Drugs of Abuse, Therapeutic Drugs, and Steroids {reprints of selected articles from the Journal of Analytical Toxicology}, 2001.

Journal Articles (Refereed)

1. Black DL, Goldberger BA, Isenschmid DS, White SM and Caplan YH: Urine cannabinoid analysis: An integrated multi-method approach. *J. Analyt. Toxicol.* 8: 224-227, 1984.
2. Goldberger BA and Caplan YH: Infrared quantitative evidential breath-alcohol analyzers: *In vitro* accuracy and precision studies. *J. Forensic Sci.* 31: 16-19, 1986.
3. Goldberger BA, Caplan YH and Zettl JR: A long-term field experience with breath ethanol collection employing silica gel. *J. Analyt. Toxicol.* 10: 194-197, 1986.
4. Goldberger BA and Caplan YH: *In vitro* accuracy and precision studies comparing direct and delayed analysis of the ethanol content of vapor. *J. Forensic Sci.* 32: 48-54, 1987.
5. Black DL, Goldberger BA and Caplan YH: Enzyme immunoassay method for comprehensive drug screening in micro-samples of urine. *Clin. Chem.* 33: 367-371, 1987.
6. Caplan YH, Levine B and Goldberger B: Fluorescence polarization immunoassay evaluated for screening for amphetamine and methamphetamine in urine. *Clin. Chem.* 33: 1200-1202, 1987.
7. Levine B, Goldberger BA and Caplan YH: Evaluation of the Coat-A-Count radioimmunoassay for phencyclidine. *Clin. Chem.* 34: 429, 1988.

8. McMullen NT, Goldberger B, Suter CM and Glaser EM: Neonatal deafening alters nonpyramidal dendrite orientation in auditory cortex: A computer microscope study in the rabbit. *J. Comp. Neurology* 267: 92-106, 1988.
9. McMullen NT, Goldberger B and Glaser EM: Postnatal development of lamina III/IV nonpyramidal neurons in rabbit auditory cortex: Quantitative and spatial analyses of golgi-impregnated material. *J. Comp. Neurology* 278: 139-155, 1988.
10. Jenkins AJ and Goldberger BA: Forensic chemistry: The analysis of controlled substances. Therapeutic Drug Monitoring and Toxicology In-Service Training and Continuing Education Program, American Association for Clinical Chemistry 13(1): 7-15, 1991.
11. Goldberger BA, Caplan YH, Maguire T and Cone EJ: Testing human hair for drugs of abuse. III. Identification of heroin and 6-acetylmorphine as indicators of heroin use. *J. Analyt. Toxicol.* 15: 226-231, 1991.
12. Levine BS, Wu SC, Goldberger BA and Caplan YH: Two fatalities involving haloperidol. *J. Analyt. Toxicol.* 15: 282-284, 1991.
13. LoDico CP, Levine BS, Goldberger BA and Caplan YH: Distribution of isoniazid in an overdose death. *J. Analyt. Toxicol.* 16: 57-59, 1992.
14. Goldberger BA and Jenkins AJ: Testing of abused drugs in urine by immunological techniques. Therapeutic Drug Monitoring and Toxicology In-Service Training and Continuing Education Program, American Association for Clinical Chemistry 13(8): 7-18, 1992.
15. Heller PF, Goldberger BA and Caplan YH: Chloral hydrate overdose: Trichloroethanol detection by gas chromatography/mass spectrometry. *Forensic Sci. International* 52: 231-234, 1992.
16. Ripple MG, Goldberger BA, Caplan YH, Blitzer MG and Schwartz S: Detection of cocaine and its metabolites in human amniotic fluid. *J. Analyt. Toxicol.* 16: 328-331, 1992.
17. Ramcharitar V, Levine BS, Goldberger BA and Caplan YH: Bupropion and alcohol fatal intoxication: Case report. *Forensic Sci. International* 56: 151-156, 1992.
18. Goldberger BA, Darwin WD, Grant TM, Allen AC, Caplan YH and Cone EJ: Measurement of heroin and its metabolites by isotope-dilution electron-impact mass spectrometry. *Clin. Chem.* 39: 670-675, 1993.
19. Klette KL, Levine B, Dreka C, Smith ML and Goldberger BA: Cholinesterase activity in postmortem blood as a screening test for organophosphate/chemical weapon exposure. *J. Forensic Sci.* 38: 950-955, 1993.

20. Cone EJ, Holicky BA, Grant TM, Darwin WD and Goldberger BA: Pharmacokinetics and pharmacodynamics of intranasal "snorted" heroin. *J. Analyt. Toxicol.* 17: 327-337, 1993.
21. Goldberger BA, Cone EJ, Grant TM, Caplan YH, Levine BS and Smialek JE: Disposition of heroin and its metabolites in heroin-related deaths. *J. Analyt. Toxicol.* 18: 22-28, 1994.
22. Jenkins AJ and Goldberger BA: New antidepressants. *Therapeutic Drug Monitoring and Toxicology In-Service Training and Continuing Education Program, American Association for Clinical Chemistry* 15: 79-87, 1994.
23. Goldberger BA and Caplan YH: Letter. The effect of glutaraldehyde (UrinAid) on the detection of abused drugs by immunoassay. *Clin. Chem.* 40: 1605-1606, 1994.
24. Goldberger BA and Cone EJ: Review. Confirmatory tests for drugs in the workplace by GC/MS. *J. Chromatogr. A* 674: 73-86, 1994.
25. Goldberger BA, Watts VW and Simonick TF: Analysis of volatiles by headspace gas chromatography. *Therapeutic Drug Monitoring and Toxicology In-Service Training and Continuing Education Program, American Association for Clinical Chemistry* 15: 231-236, 1994.
26. Goldberger BA, Loewenthal B, Darwin WD and Cone EJ: Technical brief. Intrasubject variation of creatinine and specific gravity measurements in consecutive urine specimens of heroin users. *Clin. Chem.* 41: 116-117, 1995.
27. Goldberger BA, Cone EJ and Kadehjian L: Letter to the editor. Unsuspected ethanol ingestion through soft drinks and flavored beverages. *J. Analyt. Toxicol.* 20: 332-333, 1996.
28. Roper JD, Garside D and Goldberger BA: Synthetic and semisynthetic opioid analgesics. *Therapeutic Drug Monitoring and Toxicology In-Service Training and Continuing Education Program, American Association for Clinical Chemistry* 17: 261-271, 1996.
29. Winecker RE, Goldberger BA, Tebbett I, Behnke M, Eyler FD, Conlon M, Wobie K, Karlix J and Bertholf RL: Detection of cocaine and its metabolites in amniotic fluid and umbilical cord tissue. *J. Analyt. Toxicol.* 21: 97-104, 1997.
30. Garside D, Goldberger BA, Preston KL and Cone EJ: Rapid liquid-liquid extraction of cocaine from urine for gas chromatographic-mass spectrometric analysis. *J. Chromatogr.* 692: 61-65, 1997.
31. Roper-Miller JD, Garside D and Goldberger BA: Technical brief. Automated on-line hydrolysis of benzodiazepines improves sensitivity of urine screening by a homogeneous enzyme immunoassay. *Clin. Chem.* 43:1659-1660, 1997.

32. Jenkins AJ and Goldberger BA: Identification of unique cocaine metabolites and smoking by-products in postmortem blood and urine specimens. *J. Forensic Sci.* 42:824-827, 1997.
33. Goldberger BA, Huestis MA and Wilkins DG: Commonly practiced quality control and quality assurance procedures for gas chromatography/mass spectrometry analysis in forensic urine drug-testing laboratories. *Forensic Sci. Review* 9:59-80, 1997.
34. Garside D, Ropero-Miller JD, Goldberger BA, Hamilton WF and Maples WR: Identification of cocaine analytes in fingernail and toenail specimens. *J. Forensic Sci.* 43:974-979, 1998.
35. Goldberger BA, Darraj AG, Caplan YH and Cone EJ: Detection of methadone, methadone metabolites, and other illicit drugs of abuse in hair of methadone treatment subjects. *J. Analyt. Toxicol.* 22:526-530, 1998.
36. Ropero-Miller JD and Goldberger BA: Recreational drugs: Current trends in the 90's. *Clin. Lab. Med.* 18:727-746, 1998.
37. Preston KL, Goldberger BA and Cone EJ: Occurrence of cocaine in urine of substance abuse treatment patients. *J. Analyt. Toxicol.* 22:580-586, 1998.
38. McCusker R, Paget-Wilkes H, Chronister C, Goldberger BA and ElSohly MA: Analysis of gamma-hydroxybutyrate (GHB) in urine by gas chromatography/mass spectrometry. *J. Analyt. Toxicol.* 23:301-305, 1999.
39. Preston KL, Huestis MA, Wong CJ, Umbricht A, Goldberger BA and Cone EJ: Monitoring cocaine use in substance-abuse-treatment patients by sweat and urine testing. *J. Analyt. Toxicol.* 23:313-322, 1999.
40. Hattab EM, Goldberger BA, Johannsen LM, Kindland PW, Ticino F, Chronister CW and Bertholf RL: Modification of screening immunoassays to detect sub-threshold concentrations of cocaine, cannabinoids, and opiates in urine: Use for detecting maternal and neonatal drug exposure. *Ann. Clin. Lab. Sci.* 30:85-91, 2000.
41. Ropero-Miller JD, Paget-Wilkes H, Doering PL and Goldberger BA: Effect of oral creatine supplementation on random urine creatinine, pH, and specific gravity measurements. *Clin. Chem.* 46:295-297, 2000.
42. Ropero-Miller JD, Goldberger BA, Cone EJ and Joseph Jr, RE: The disposition of cocaine and opiate analytes in hair and fingernails of humans following cocaine and codeine administration. *J. Analyt. Toxicol.* 24:496-508, 2000.
43. Caplan YH and Goldberger BA. Alternative Specimens for Workplace Drug Testing: *J. Analyt. Toxicol.* 25:396-399, 2001.

44. Winecker RE, Goldberger BA, Tebbett IR, Behnke M, Eyler FD, Karlix JL, Wobie K, Conlon M, Phillips D and Bertholf RL: Detection of Cocaine and Its Metabolites in Breast Milk. *J. Forensic Sci.* 46:1221-1223, 2001.
45. Chronister CW, Walrath JC and Goldberger BA: Rapid Detection of Benzoylcegonine in Vitreous Humor by Enzyme Immunoassay. *J. Analyt. Toxicol.* 25:621-624, 2001.
46. Goldberger BA and Polettini A: Forensic Toxicology: Web Resources. *Toxicology* 173:97-102, 2002.
47. Murray RA, Doering PL, Boothby LA, Merves ML, McCusker RR, Chronister CW and Goldberger BA: Putting an Ecstasy Test Kit to the Test – Harm Reduction or Harm Induction? *Pharmacotherapy* 23: 1238-1244, 2003.
48. McCusker RR, Goldberger BA and Cone EJ: Caffeine Content of Specialty Coffees. *J. Analyt. Toxicol.* 27:520-522, 2003.
49. Szabo ST, Gold MS, Goldberger BA and Blier P: Effects of Sustained Gamma-Hydroxybutyrate Treatments on Spontaneous and Evoked Firing Activity of Locus Coeruleus Norepinephrine Neurons. *Biol. Psychiatry* 55: 934-939, 2004.
50. Drummer O, Forrest ARW, Goldberger B and Karch SB. Forensic Science in the Dock *British Med J.* 329:636-637, 2004.
51. Li M, Goldberger BA and Hopkins C: Fatal Case of Botox®-Related Anaphylaxis? A Case Report. *J. Forensic Sci.* 50:169-172, 2005.
52. McCusker RR, Goldberger BA and Cone EJ: Caffeine Content of Energy Drinks, Carbonated Sodas and Other Beverages. *J. Analyt. Toxicol.* 30:112-114, 2006.
53. McAuliffe PF, Gold MS, Bajpai L, Merves ML, Frost-Pineda K, Pomm RM, Goldberger BA, Melker RJ and Cendan JC: Second-hand exposure to aerosolized intravenous anesthetics propofol and fentanyl may cause sensitization and subsequent opiate addiction among anesthesiologists and surgeons. *Med Hypotheses.* 66: 874-882, 2006.
54. O'Dell LE, Bruijnzeel AW, Smith RT, Parsons LH, Merves ML, Goldberger BA, Richardson HN, Koob GF and Markou A. Diminished nicotine withdrawal in adolescent rats: implications for vulnerability to addiction. *Psychopharmacology.* 186:612-619, 2006.
55. Gold MS, Frost-Pineda K, Goldberger BA and DuPont RL. Letter to the Editor – Physicians and Drug Screening. *J. Adolesc. Health.* 39: 154-155, 2006.
56. McCusker RR, Fuehrlein B, Goldberger BA, Gold MS and Cone EJ: Caffeine Content of Decaffeinated Coffee. *J. Analyt. Toxicol.* 30:611-3, 2006.

57. Gold MS, Kolodner D, Logan D and Goldberger BA. Response by Mark Gold et al. *Journal of Addictive Diseases*. 25:141-144, 2006.
58. Chronister CW, Gascoigne A and Goldberger BA. Rapid Detection of Opioids in Vitreous Humor by Enzyme Immunoassay. *J. Analyt. Toxicol.* (in press).

Proceedings

1. Goldberger BA and Caplan YH: Evaluation of the adsorption and desorption of ethanol from breath specimens using silica gel. The International Symposium on Driving Under the Influence of Alcohol and/or Drugs, Federal Bureau of Investigation, Quantico, Virginia, pp. 119-121, 1986.
2. Caplan YH, Goldberger BA and Physioc GE: Comparison of a Breathalyzer Model 900B prototype with the Breathalyzer Model 900A. 11th International Conference on Alcohol, Drugs and Traffic Safety, National Safety Council, Chicago, Illinois, pp. 766-768, 1991.
3. Caplan YH, Levine BS and Goldberger BA: Drugs in driver fatalities: A preliminary study in the state of Maryland. 11th International Conference on Alcohol, Drugs and Traffic Safety, National Safety Council, Chicago, Illinois, pp. 824-828, 1991.
4. Cone EJ and Goldberger BA: Pharmacokinetics and tissue distribution of opiates. Drug Abuse in the Decade of the Brain, Symposium for Health and Substance Abuse Professionals, Houston, Texas, pp. 71-77, 1997.

Articles (Not Refereed)

1. Isenschmid DS, Goldberger BA, Caplan YH and Straus PW: GC-MS automation for drug testing laboratories. Hewlett-Packard Application Note, GCMS 89-3, 1989.
2. Goldberger BA: Heroin. *Therapeutic Drug Monitoring and Toxicology*, American Association for Clinical Chemistry 12(12): 3-4, 1991.
3. Goldberger BA: Bupropion. *Therapeutic Drug Monitoring and Toxicology*, American Association for Clinical Chemistry 13(8): 3-4, 1992.
4. Jenkins AJ, Goldberger BA, Caplan YH and Hoey LD: GC/MS confirmation of benzoylecgonine in blood, plasma and urine specimens. Hewlett-Packard Application Note, GCMS 92-01, 1992.
5. Goldberger BA: Cocaethylene. *Therapeutic Drug Monitoring and Toxicology*, American Association for Clinical Chemistry 13(16): 3-4, 1992.
6. Jenkins AJ, Goldberger BA, Cone EJ and Hoey LD: GC/MS Assay of 6-acetylmorphine in plasma. Hewlett-Packard Application Note, GCMS 93-04, 1993.

7. Goldberger BA: Lysergic acid diethylamide. Therapeutic Drug Monitoring and Toxicology, American Association for Clinical Chemistry 14: 99-100, 1993.
8. Goldberger BA: Clonidine. Therapeutic Drug Monitoring and Toxicology, American Association for Clinical Chemistry 15: 3-4, 1994.
9. Goldberger BA: Methadone. Therapeutic Drug Monitoring and Toxicology, American Association for Clinical Chemistry 15: 155-156, 1994.
10. Goldberger BA: Venlafaxine. Therapeutic Drug Monitoring and Toxicology In-Service, American Association for Clinical Chemistry 16: 3-4, 1995.
11. Garside D and Goldberger BA: Nefazodone. Therapeutic Drug Monitoring and Toxicology, American Association for Clinical Chemistry 16: 109-110, 1995.
12. Goldberger BA and Huestis MA: GC/MS: Quality assurance/quality control procedures for forensic urine drug testing laboratories. Hewlett-Packard Application Note 228-322, July, 1995.
13. Garside D and Goldberger BA: Zolpidem. Therapeutic Drug Monitoring and Toxicology, American Association for Clinical Chemistry 16: 193-195, 1995.
14. Goldberger BA and Miles WS: Benzoyllecgonine in urine: Validating a PrepStation/GC/MSD system that integrates automated sample preparation with analysis. Hewlett-Packard Application Note 228-338, October, 1995.
15. Garside D and Goldberger BA: Ranitidine. Therapeutic Drug Monitoring and Toxicology, American Association for Clinical Chemistry 16: 277-279, 1995.
16. Goldberger BA and Miles WS: THC-acid in urine: Validating a PrepStation/GC/MSD system that integrates automated sample preparation with analysis. Hewlett-Packard Application Note 228-345, December 1995.
17. Goldberger BA and Miles WS: PCP in urine: Validating a PrepStation/GC/MSD system that integrates automated sample preparation with analysis. Hewlett-Packard Application Note 228-346, December, 1995.
18. Goldberger BA and Miles WS: Opiates in urine: Validating a PrepStation/GC/MSD system that integrates automated sample preparation with analysis. Hewlett-Packard Application Note 228-347, December, 1995.
19. Garside D and Goldberger BA: Valproate - A new treatment for bipolar disorder. Therapeutic Drug Monitoring and Toxicology, American Association for Clinical Chemistry 17: 55-56, 1996.
20. Goldberger BA: What the Experts Think - Improving Drug Detection in the Workplace

through the Analysis of Alternative Specimens. Clin Lab News 25: 12:1999.

Abstracts

1. Goldberger BA and Caplan YH: An *in vitro* accuracy and precision study of four quantitative breath alcohol analyzers. Mid-Atlantic Association of Forensic Scientists, Washington, D.C., 1985.
2. Goldberger BA and Caplan YH: The Breathalyzer 900/900A: Breath collection and delayed alcohol analysis. Mid-Atlantic Association of Forensic Scientists, Washington, D.C., 1985.
3. Goldberger BA and Caplan YH: Delayed vapor alcohol analysis using silica gel: accuracy, precision, stability and field studies. American Academy of Forensic Sciences, New Orleans, Louisiana, 1986.
4. Goldberger BA and Caplan YH: Evaluation of the adsorption and desorption of ethanol from breath specimens using silica gel. The International Symposium on Driving Under the Influence of Alcohol and/or Drugs, Federal Bureau of Investigation, Quantico, Virginia, 1986.
5. Goldberger BA and Caplan YH: Experiences with silica gel trapping of alcohol in breath testing. American College of Toxicology, Baltimore, Maryland, 1987.
6. Goldberger BA: Automation for forensic urine drug testing laboratories. Scientific Computing and Automation, Conference and Exposition, Philadelphia, Pennsylvania, 1989.
7. Isenschmid DS, Goldberger BA, Harbin GF and Caplan YH: Customizing the HP-1000 GC/MS data system for automated data analysis. Scientific Computing and Automation, Conference and Exposition, Philadelphia, Pennsylvania, 1989.
8. Caplan YH, Levine BS and Goldberger BA: Drugs in driver fatalities: A preliminary study in the state of Maryland. International Conference on Alcohol, Drugs and Traffic Safety, Chicago, Illinois, 1989.
9. Caplan YH, Goldberger BA and Physioc GE: Comparison of a Breathalyzer Model 900B prototype with the Breathalyzer Model 900A. International Conference on Alcohol, Drugs and Traffic Safety, Chicago, Illinois, 1989.
10. Goldberger BA, Caplan YH, Maguire T and Cone EJ: Detection of heroin and 6-acetylmorphine in hair. Society of Forensic Toxicologists, Montreal, Canada, 1991.
11. Cone EJ and Goldberger BA: Decontamination issues in testing hair for cocaine and heroin. Society of Forensic Toxicologists, Montreal, Canada, 1991.

12. Goldberger BA, Caplan YH, Darwin WD and Cone EJ: Assay development for heroin and metabolites by gas chromatography/mass spectrometry. American Academy of Forensic Sciences, New Orleans, Louisiana, 1992.
13. Klette KL, Levine B, Smith M, Goldberger B and Dreka C: Cholinesterase activity in postmortem blood, as an indication of chemical weapon exposure. American Academy of Forensic Sciences, New Orleans, Louisiana, 1992.
14. Goldberger BA, Grant TM, Darwin WD, Jenkins AJ, Cone EJ and Caplan YH: Identification of heroin, 6-acetylmorphine and morphine in post mortem urine by GC/MS. Society of Forensic Toxicologists, Cromwell, Connecticut, 1992.
15. Jenkins AJ, Goldberger BA, Darwin WD and Cone EJ: Identification of unique cocaine metabolites and smoking by-products in postmortem blood and urine specimens. American Academy of Forensic Sciences, Boston, Massachusetts, 1993.
16. Goldberger BA, Caplan YH and Cone EJ: Measurement and interpretation of heroin, 6-acetylmorphine and morphine concentrations in biological tissues obtained from heroin users and heroin-related deaths. Society of Forensic Toxicologists, Phoenix, Arizona, 1993.
17. Goldberger BA, Caplan YH and Miles WS: Automated solid-phase extraction and GC/MS analysis of benzoylecgonine in urine. American Academy of Forensic Sciences, San Antonio, Texas, 1994.
18. Goldberger BA, Cone EJ and Caplan YH: Significance of heroin and 6-acetylmorphine in hair. 2nd International Meeting on Clinical and Forensic Aspects of Hair Analysis, Genova, Italy, 1994.
19. Goldberger BA, Darraj AG, Cone EJ and Caplan YH: Detection of methadone, methadone metabolites, and other illicit drugs of abuse in hair of methadone treatment Subjects. 2nd International Meeting on Clinical and Forensic Aspects of Hair Analysis, Genova, Italy, 1994.
20. Garside D and Goldberger BA: "CyberTox": Toxicology resources on the Internet. Society of Forensic Toxicologists, Baltimore, Maryland, 1995.
21. Goldberger BA and Garside D: Toxicological investigation of the Jesse James exhumation. American Academy of Forensic Sciences, Nashville, Tennessee, 1996.
22. Winecker RE, Tebbett I, Goldberger BA and Bertholf RL: Utility of maternal hair for determining prenatal cocaine exposure. American Association for Clinical Chemistry, Chicago, Illinois, 1996.
23. Roper JD, Garside D, Goldberger BA, Smialek JE, Zielke RH and Vigorito R: Preliminary findings of hair analysis in SIDS-Related deaths. Society of Forensic Toxicologists, Denver, Colorado, 1996.

24. Goldberger BA and Caplan YH: The C.I.A., LSD and the Strange death of Frank R. Olson, Ph.D.: What the June 1994 exhumation hath wrought!; Toxicological analyses. American Academy of Forensic Sciences, New York City, New York, 1997.
25. Preston KL, Silverman K, Goldberger BA and Cone EJ: Criteria for identifying new cocaine use: Presence of cocaine in urine. American Society for Clinical Pharmacology and Therapeutics, San Diego, California, 1997.
26. Ropero-Miller JD, Goldberger BA, Vance R and Kintz P: Implementation of the State of Florida hair analysis proficiency testing program. Society of Forensic Toxicologists, Salt Lake City, Utah, 1997.
27. Goldberger BA: The Death of J. Edgar Hoover: A mystery in search of a solution – Toxicological considerations. American Academy of Forensic Sciences, San Francisco, California, 1998.
28. Ropero-Miller JD, Garside D, Goldberger BA, Hamilton WF and Maples WR: Nails: Another useful matrix for the identification of cocaine exposure. American Academy of Forensic Sciences, San Francisco, California, 1998.
29. Winecker RE, Goldberger BA, Tebbett IR, Behnke M, Eyler FD, Karlix J, Wobie K, Phillips D and Bertholf RL: Cocaine in breast milk: The potential for postnatal cocaine exposure. American Academy of Forensic Sciences, San Francisco, California, 1998.
30. Logan BK, Blaho K, Mandrell T, Berryman H, Goff ML, Goldberger B, Schwilke G and Ropero-Miller JD: Effects of death and decomposition on concentrations of cocaine and metabolites in juvenile swine. American Academy of Forensic Sciences, San Francisco, California, 1998.
31. Ropero-Miller JD, Goldberger BA and Doering PL: Oral creatine supplementation and its effect on urinary creatinine, pH, and specific gravity measurements. Joint meeting of the Society of Forensic Toxicologists and The International Association of Forensic Toxicologists, Albuquerque, New Mexico, 1998.
32. Goldberger BA: Forensic Colloquia (Jurisprudence Section) – The Melinda McIntosh exhumation; Toxicological analysis. American Academy of Forensic Sciences, Orlando, Florida, 1999.
33. Ropero-Miller JD, Goldberger BA, Oyler JM, Cone EJ and Joseph Jr, RE: Drug testing with alternative matrices: Cocaine disposition in hair and fingernails. American Academy of Forensic Sciences, Orlando, Florida, 1999.
34. Bertholf RL, Goldberger BA, Johannsen LM, Chronister CW and Hattab E: Use of CEDIA screening methods for cocaine metabolite, cannabinoids, and opiates at sub-threshold concentrations: Improved sensitivity in clinical applications. American Academy of Forensic Sciences, Orlando, Florida, 1999.

35. McCusker RR, Paget-Wilkes H, Chronister C, Goldberger BA and ElSohly MA: Analysis of gamma-hydroxybutyrate (GHB) in urine by gas chromatography/mass spectrometry. Society of Forensic Toxicologists, Puerto Rico, 1999.
36. Chronister CW, Walrath JC and Goldberger BA: Detection of cocaine/cocaine Metabolite in vitreous humor by cloned enzyme donor immunoassay. Society of Forensic Toxicologists, Milwaukee, Wisconsin, 2000.
37. Goldberger BA, Frost-Pineda K and Gold MS. Ecstasy deaths in the State of Florida: A post-mortem analysis. Society of Biological Psychiatry, Philadelphia, Pennsylvania, 2002.
38. Goldberger BA, Frost-Pineda K and Gold MS: Methadone overdose deaths in the State of Florida: A post-mortem analysis. American College of Clinical Pharmacology, San Francisco, California, 2002.
39. Szabo ST, Gold MS, Goldberger BA, Blier P. Effects of sustained gamma-hydroxybutyrate (GHB) treatments on spontaneous and evoked firing activity of locus coeruleus neurons. Society of Neuroscience, Orlando, Florida, 2002.
40. Murray RA, Doering PL, Merves ML, McCusker RR, Chronister CW and Goldberger BA: Putting an ecstasy test kit to the test. American Academy of Forensic Sciences, Chicago, Illinois, 2003.
41. Merves ML, McCusker RR, Chronister CW and Goldberger BA: Analysis of barbiturates by fast GC: A preliminary study. American Academy of Forensic Sciences, Chicago, Illinois, 2003.
42. Goldberger BA, Frost-Pineda K, Gold MS. Methadone related deaths exceed heroin in the State of Florida. American Society for Addiction Medicine. Toronto, Canada, 2003.
43. Goldberger BA, Lessig MC, McCusker RR, Cone EJ and Gold MS. Evaluation of current caffeine content of coffee beverages: Recommendations for clinicians regarding caffeine exposure. Society of Biological Psychiatry, San Francisco, California, 2003.
44. Goldberger BA, Merves ML, Frost-Pineda K, Gold MS. The use and abuse of prescription opioids: An epidemic of deaths in the State of Florida. College on Problems of Drug Dependence. Bal Harbour, Florida, 2003.
45. Szabo ST, Gold MS, Goldberger BA, Blier P. Effects of sustained gamma-hydroxybutyrate (GHB) treatments on spontaneous and evoked firing activity of locus coeruleus norepinephrine neurons. American College of Clinical Pharmacology. Palm Harbor, Florida, 2003.

46. Goldberger BA, Merves ML, Frost-Pineda K, Gold MS. Use, misuse and diversion of methadone and hydrocodone: The deaths continue to increase. American College of Clinical Pharmacology. Palm Harbor, Florida, 2003.
47. McCusker RR, Merves ML, Goldberger BA and Cone EJ. Café Noir, Café Au Lait, or just caffeine? Society of Forensic Toxicologists, Portland, Oregon, 2003.
48. Langee CL, Goldberger BA and Martin JV. Postmortem morphine concentrations – Are they meaningful? American Academy of Forensic Sciences, Dallas, Texas, 2004.
49. Li M, Goldberger BA and Hopkins C. Fatal Botox-induced anaphylaxis? A case report. American Academy of Forensic Sciences, Dallas, Texas, 2004.
50. Goldberger BA, Frost-Pineda K, Gold MS. Methadone related deaths exceed heroin in the State of Florida. American Society for Addiction Medicine. New York, New York, 2004.
51. Goldberger BA, McCusker RR, Byars JA, Frost-Pineda K and Gold MS. Decaffeinated coffee: An oxymoron. Society of Biological Psychiatry, New York, New York, 2004.
52. Merves ML, Chronister CW, Goldberger BA, Melker RJ and Gold MS. Detection of cotinine in exhaled breath by gas chromatography-mass spectrometry – A preliminary study. Society of Forensic Toxicologists, Washington, D.C., 2004.
53. Goldberger BA, Melker RJ, Merves ML, Frost-Pineda K and Gold MS. Measuring and quantifying substance abuse: cotinine in exhaled breath. Society for Neuroscience, San Diego, California, 2004.
54. Fuehrlein B, Goldberger BA, McCusker RR, Warren MW and Gold MS. The addictive potential of decaffeinated coffee. Society for Neuroscience, San Diego, California, 2004.
55. Goldberger BA, Fileger MI, Merves ML and Gold MS. Fentanyl use and abuse: New emerging trends in drug abuse. College on Problems of Drug Dependence. Orlando, Florida, 2005.
56. McCusker RR, Merves ML, Goldberger BA and Cone EJ. Cafe Noir, Cafe Au Lait, or just caffeine? – Part 2. Society of Forensic Toxicologists, Nashville, Tennessee, 2005.
57. Goldberger BA, Merves ML, MacDougall KN, Chronister CW, Martínez MA and Nelson SJ. Paraquat in exhumed remains by gas chromatography-mass spectrometry. Society of Forensic Toxicologists, Nashville, Tennessee, 2005.
58. Merves ML, Grosskopf TD, Chronister CW and Goldberger BA. Detection of benzoylecgonine in urine using the V-Flex system. American Academy of Forensic Sciences, Seattle, Washington, 2006.

59. Bedore LR, Goldberger BA and Lonesk KH. FEMORS – The State of Florida’s mass fatality response team. American Academy of Forensic Sciences, Seattle, Washington, 2006.
60. Kero FA, Booth MM, Dennis DM, Goldberger BA, Conner GE and Yost RA. Development of a high-throughput SPE-LC-MS/MS method to quantify trace levels of endogenous thiocyanate in complex biological fluids. American Society for Mass Spectrometry, Seattle, Washington, 2006.
61. Cone EJ, Schnoll S, Pinney J, Henningfield JE, Fant RV, Goldberger BA and Chronister CW. Internet monitoring: getting high by pharmaceutical tampering. The International Association of Forensic Toxicologists, Ljubljana, Slovenia, 2006.
62. Goldberger BA, Merves ML, Gres K, Melker RJ, Goodman WK, Gold MS. Further Evidence of Second-hand Exposure to Drugs in the Operating Room. Society for Neuroscience, Atlanta, Georgia, 2006.
63. Cunningham MA, Goldberger BA, Graham NA and Gold MS, Cocaine – A Recurring Epidemic in the Florida? Society for Neuroscience, Atlanta, Georgia, 2006.
64. Cunningham MA, Graham NA, Goldberger BA and Gold MS, Cocaine Abuse in 2006 – A New Epidemic. American Academy of Addiction Psychiatry, St. Pete Beach, Florida, 2006.

March 27, 2007

Curriculum Vitae

Allen C. Lim, PhD

April 2007

Date of Birth: December 1, 1972

Place of Birth: Davao City, Philippines

Business Address:

1118 13th Street, #A30

Boulder, CO 80302

303-396-8845

E-mail: allen@slipstreamsports.com

Home Address:

770 Walnut, Unit D

Boulder, CO 80302

720-938-3094

E-mail: allenlim@mac.com

Education:

1998-2004

University of Colorado at Boulder

Department of Integrative Physiology

Doctor of Philosophy, Applied Exercise Science

1994-1996

University of Colorado at Boulder

Department of Kinesiology

Master of Science, Kinesiology

1990-1994

University of California at Davis

Department of Physical Education

Bachelor of Science, Exercise Science

Professional Experiences:

2006-Present

Sports Physiologist: Slipstream Sports Professional Cycling Team. Boulder, Colorado & Gerona, Spain.

2005-2006

Sport Science Consultant: Phonak Professional Cycling Team. Switzerland.

2005-Present

President & Founder: 99 One, Inc. Sports Science & Consulting Services. Boulder, Colorado.

2003-Present

Director of Education: Saris Cycling Group, manufacturers of the CycleOps PowerTap. Madison, Wisconsin.

2005-2006

Sport Science Consultant: Phonak Professional Cycling Team

1998-2000

Consultant: E-Tune, Developers of the PowerTap rear hub power meter. Boston, Massachusetts.

1998

Wellness Consultant: Center for Creative Leadership, Colorado Springs, Colorado.

1998-2004

Exercise Test Technologist: University of Colorado

1994-1996

Community Wellness and Education Program. Boulder, Colorado.

Academic Appointments:

2002

Instructor: Exercise Physiology, CU Boulder

2002-2003

Teaching Assistant: Statistics, CU Boulder

2002-2003

Teaching Assistant: Nutrition, CU Boulder

1995-1998

Instructor: "Electrocardiography & Exercise Stress Testing"

| | |
|-----------------------|---|
| Fall 2000-Spring 2001 | University/Community Wellness and Education Program. <i>Lab Director & Teaching Assistant:</i> Exercise Physiology, CU Boulder |
| Fall 1998 | <i>Teaching Assistant:</i> Neuromuscular Physiology, CU Boulder. |
| Fall 1998 | <i>Teaching Assistant:</i> Statistics, CU Boulder. |
| Fall 1995-Spring 1996 | <i>Teaching Assistant:</i> Exercise Physiology, CU Boulder. |
| Spring 1995 | <i>Laboratory Assistant:</i> Human Performance Lab, CU Boulder. |

Grants and Fellowships Received:

| | |
|---------------------|--------------------------------------|
| 2001-2004 | Saris Cycling Group Research Grant. |
| Spring 00 – Fall 01 | Dean's Fellowship Award. |
| Fall 99 – Spring 00 | Dean's Fellowship Award. |
| Fall 1999 | University Fellowship Award. |
| Spring 1999 | Celestial Seasonings Research Grant. |
| Spring 1996 | Deans Small Grant. |
| Fall 1995 | Graduate School Fellowship Award. |

Research Experience:

| | |
|-------------|--|
| 2003-2004 | <i>Primary Investigator:</i> "Physiological vs. physical determinants of uphill vs. level time trial performance." CU Boulder. |
| 2002-2003 | <i>Primary Investigator:</i> "Predicting aerodynamic drag using a rear hub power meter." CU Boulder. |
| 2000 | <i>Primary Investigator:</i> "Heart Rate vs. Power in men and women during a National Calendar stage race." CU Boulder |
| 2004 | <i>Co-Investigator:</i> "Cycling performance at moderate altitude versus sea level simulated with a hyperoxic gas." CU Boulder. |
| 2000-2004 | <i>Co-Investigator:</i> "Bone density in professional cyclists." CU Boulder. |
| 1999-Date | <i>Co-Investigator:</i> "The influence of pedal cadence on uphill cycling performance and economy." CU Boulder. |
| 1999-Date | <i>Co-Investigator:</i> "Maximal sustainable power at fixed percentages of peak power output." CU Boulder. |
| 1998-Date | <i>Primary Investigator:</i> "The physical and physiological demands of competition and training in elite cyclists." CU Boulder. |
| 1999 | <i>Co-Investigator:</i> "The reliability and validity of the Power Tap® rear bicycle hub dynonameter." CU Boulder. |
| Summer 1997 | <i>Research Assistant:</i> "Work fitness of commercial porters of Eastern Nepal." CU Boulder. |
| 1995-1996 | <i>Primary Investigator:</i> "Exercise induced desaturation in elite female cyclists." Department of Kinesiology, CU Boulder & The USOC Sport Science & Technology Division, Colorado Springs, Colorado. |
| 1994 | <i>Lab Assistant:</i> "The relationship of pulmonary ventilation and heart rate in arm work alone, leg work alone, and in various combinations of arm and leg work when exposed to moderate and hot ambient temperatures." Investigators: Adams, W.C., & J.D. Shaffrath, Department of Exercise Science, University of California at Davis. |

Publications:

| | |
|-----------|---|
| In Review | Lim, A.C., Carver, T.C., Edwards, A.G., Byrnes, W.C. "Physiological vs. physical determinants of uphill vs. level time trial performance." <i>Medicine & Science in Sports & Exercise</i> . |
| In Review | Edwards, A.G., Lim, A.C., and Byrnes, W.C. "Predicting aerodynamic drag using a rear hub power meter." <i>Medicine & Science in Sports & Exercise</i> . |
| In Review | Turner, B.T., Lim, A.C., and Byrnes, W.C. "The influence of pedal cadence on uphill cycling performance and economy." <i>Medicine & Science in Sports & Exercise</i> . |
| In Review | Ziewacz, D.C., Lim, A.C., and Byrnes, W.C. "The reliability and validity of the Power Tap® rear bicycle hub dynamometer." <i>Medicine & Science in Sports & Exercise</i> . |
| 2001 | Malville, NJ, Byrnes WC, Lim, AC, Basnyat, R. Commercial porters of eastern Nepal: health status, physical work capacity, and energy expenditure. <i>American Journal of Human Biology</i> . Vol 13, No. 1, pp. 44-56. Jan 2001. |
| 1998 | Lim, A.C. Heat and cold stress in competitive cycling. United States Cycling Federation Elite Coaching Manual. 1998. |
| 1997 | Lim, A.C., W.C. Byrnes, R.L. Wilber, and J.T. Kearney. Exercise-induced desaturation in elite female and male cyclists at moderate altitudes. Abstract. <i>Medicine & Science In Sports & Exercise</i> . Vol. 29, No. 5, P. S286, May 1997. |

Certifications & Memberships:

| | |
|-----------|--|
| 1995-Date | <i>Exercise Test Technologist:</i> ACSM Certification # 3840 |
| 1998-Date | <i>Member:</i> American College of Sports Medicine |

Coaching Experience:

| | |
|-----------|---|
| 2007 | <i>Coach:</i> Slipstream-Chipotle Professional Cycling Team 3 rd place, World Track Championship Omnium Most Aggressive Rider, Tour of California |
| 2006 | <i>Sports Physiologist & Training Advisor:</i> Floyd Landis, Winner of the 2006 Tour de France. |
| 2006 | <i>Coach:</i> TIAA-CREF Professional Cycling Team 1 st place, Under 23 National Road Cycling Championships 1 st place, Under 23 National Criterium Cycling Championships 1 st place, US Professional Criterium Championships 3 rd place, US Professional Road Championships 1 st place, Team Pursuit, National Track Championships 1 st place, Points Race, National Track Championships 1 st place, Madison, National Track Championships 1 st place, Individual Pursuit, National Track Championships |
| 2005 | <i>Sports Physiologist & Training Advisor:</i> Floyd Landis, 9 th place in the 2005 Tour de France. |
| 2000-2004 | <i>Personal Coach & Advisor:</i> Michael Barry, 2005 Canadian Olympic Team Member Dede Barry, 2004 US Olympic Team Member, Silver Medalist Colby Pearce, 2004 US Olympic Team Member |

| | |
|-----------|--|
| 1999 | Christian VandeVelde, 2000 US Olympic Team Member Scott Moninger, US Record Holder for Total Career Wins Alex Candelario, Collegiate National Road Champion <i>Director/Coach:</i> Celestial Seasonings Women's Cycling Team 1 st Place, Pan American Time Trial Championships 1 st Place, Pan American Road Race Championships 1 st Place, US National Criterium Championships Top American, First Union World Cup, Philadelphia, PA Top American, World Time Trial Championships Top American, World Road Race Championships |
| 1998 | <i>Director/Coach:</i> Celestial Seasonings Women's Cycling Team. 1 st Place, National Time Trial Championships 1 st Place, Under 23 National Time Trial & Road Championships 1 st Place, 1998 Junior National Criterium, Road, & Time Trial Championships (16-17 yrs) |
| 1997 | <i>Resident Coach:</i> U.S. National Cycling Team U.S. Olympic Training Center, Colorado Springs, Colorado. |
| 1994-1995 | <i>Head Coach:</i> CU Boulder Women's Cycling Team 3rd Place, 1995 Collegiate Nationals |
| 1993-1994 | <i>Head Coach:</i> U.C. Davis Cycling Team 1st Place, 1994 Collegiate Nationals |

Public Appearances and Presentations:

| | |
|-------------------------|---|
| Winter-Spring 2005-2006 | <i>Featured Speaker:</i> Nation wide lecture tour examining the power demands of Floyd Landis during the 2005 Tour De France. Sponsored by the Saris Cycling Group. |
| June 7, 2005 | <i>Slide Presentation:</i> "Physiological vs. physical determinants of uphill vs. level time trial performance." American College of Sports medicine 52 nd Annual Meeting. |
| April, 25 2005 | <i>Guest Speaker:</i> "Redefining road cycling performance from field measurements of power." University of Wisconsin, Madison, Department of Mechanical Engineering. |
| July 12, 1999 | <i>Interview & Feature:</i> "Sport Science & Technology in Cycling." <i>NBC Wide World of Sports</i> , HP Women's Challenge. Pioneered use of a rider mounted telemetry system with Tune Inc., that allowed direct video capture of power output, speed, heart rate, and distance from the bicycle. |
| May 31, 1997 | <i>Poster Presentation:</i> "Exercise Induced Desaturation in Elite Female and Male Cyclists." American College of Sports Medicine 44 th Annual Meeting. |

Other:

| | |
|-------------|---|
| Summer 1996 | Advisor & Guest of the Chinese General Consulate. Games of the XXVI Olympiad, Atlanta 1996. |
|-------------|---|

Dr. Wolfram Meier-Augenstein, CChem, MRSC

Senior Lecturer

Environmental Forensics & Human Health Lab.

Environmental Engineering Research Centre

David Keir Building, 39-123 Stranmillis Road

Queen's University Belfast

BELFAST

BT9 5AG

Tel.: 028 – 9097 4015

Fax: 028 – 9066 3754

e-mail: w.meier-augenstein@qub.ac.uk

Career:

- 01.02.07 to date: Senior Lecturer, Environmental Engineering Research Centre; School of Planning, Architecture & Civil Engineering, Queen's University Belfast
- 01.10.05-31.01.07: Lecturer (B), Environmental Engineering Research Centre; School of Planning, Architecture & Civil Engineering, Queen's University Belfast.
- 01.01.03-30.09.05: Research Officer / Senior Research Fellow, Environmental Engineering Research Centre; Civil Engineering, Queen's University Belfast.
- 01.02.99-31.12.02: Senior Research Fellow, Division of Molecular Physiology, School of Life Sciences; University of Dundee. Radiation Protection Supervisor.
- 01.10.97-31.01.99: Research Fellow / Lecturer (Chemistry), Metabolic Studies Group, Dept of Anatomy & Physiology; University of Dundee, UK.
- 01.10.94-30.09.97: Research Fellow / Hon. Lecturer (Chemistry), Metabolic Studies Group, Dept. of Anatomy & Physiology; University of Dundee, UK.
- 01.02.92-30.09.94: Research Assistant, Metabolic Disorders Screening Lab., Dept. of Paediatric Neurology, University Children's Hospital, University of Heidelberg, Germany.
- 01.06.92-31.08.92: Visiting Scientist at Prof. W. N. Nyhan's Biochemical Genetics Lab., University of California San Diego, USA.
- 01.01.90-30.04.91: Post-Doctoral Fellow, Ecological Biochemistry Group, Dept. of Organic Chemistry, University of Stellenbosch, South Africa.
- 01.09.87-31.12.89: Doctoral Fellow, Natural Compound Chemistry Group, Dept. of Organic Chemistry; Radiation Protection Officer; University of Heidelberg, Germany.

Qualifications:

- PhD (1989), *magna cum laude* (mark 1.0), University of Heidelberg, Germany: Title of Thesis: "A PLMF-1 specific receptor in *Mimosa pudica* L."
- Dipl. Chem. (1987), 'good' (mark 1.9), in Chemistry and Molecular Genetics; University of Heidelberg, Germany; Title of Thesis: "A new synthetic pathway for 4-O-(β -D-glucopyranosyl)gallic acid (PLMF-1)."
- Radiation Protection Officer (cert.) (1987), Nuclear Research Centre Karlsruhe, Germany.

Awards:

- Feodor-Lynen Fellowship awarded by the Alexander-von-Humboldt Stiftung, Bonn, Germany; November 1990.
- Post-Doctoral Fellowship awarded by the Foundation for Research Development, Pretoria, South Africa; January 1990.

Research Interests:

- Study of stable isotope profiles and reaction specific isotopic fractionation factors of (bio-)organic compounds for forensic analysis of source, origin and authenticity.
- Study of biochemical transformation of exogenously and endogenously sourced amino acids into keratin and collagen as well as study of metabolic fate, turnover rates and pathways of incorporation of light and trace elements into human hair, nails and bone for forensic determination of geographic point of origin and geographic movement of living people and human remains.
- Design and development of pathway-specific stable isotope labelled probes for direct measurement of (patho-)physiological and metabolic processes to measure environmental impact on human health and for therapy control.
- Compound specific study of volatile organic compounds in human breath and indoor air to determine their origin and relevance as indicators of exposure and/or disease.
- Stable isotope profiling of airborne organic pollutants and contaminants in the environment to determine their age, distribution and point of origin as well as uptake by and metabolic fate in the human body.

Current Grants / Projects:

- Procter & Gamble funded research project (PI) on "Isotope Fingerprinting of Authentic and Counterfeit Goods for both Brand and Consumer Protection"; \$25,000; March 2007 to February 2008.
- EPSRC Think Crime 4th Call grant (PI) with Dr N NicDaeid (CFS, University of Strathclyde): "Isotope Profiling of Drugs to Combat Serious and Organised Crime"; £107,023; February 2006 to January 2009.
- RSC/EPSRC PhD Studentship; "Multi-dimensional Profiling of Drugs and Fibres"; £60,190; October 2006 to March 2010.
- PhD studentship award made by the Defence Science & Technology Laboratory (dstl), Fort Halstead (UK); £29,393; January 2006 – December 2008.
- Invest NI Proof of Concept grant (PI): "Non-invasive functional screening for reduced liver function and adverse drug effects"; February 2005 to July 2006; £108,010.
- EPSRC Platform grant "Environmental Forensics" as Co-Investigator together with Prof RM Kalin (PI) and Dr T Elliot (Co-I); August 2004 to July 2009; £448,624.

Completed Grants / Research Contracts:

- Research project (PI) for longitudinal variation and validation of my SAFEBT Gastric Emptying Breath Test (pat. pending) in adult patients; Johnson & Johnson PRD; April 2004 to October 2005; £62,000.
- Research project (PI) for patient screening and randomisation using my SAFEBT Gastric Emptying Breath Test (pat. pending) in a multi-centre clinical trial setting; Johnson & Johnson PRD Inc; January 2003 to December 2003; £160,400.
- World Anti-Doping Agency (WADA) joint grant application with Prof. M.J. Rennie (PI) (University of Nottingham): "A novel method to detect endogenous and pharmaceutical androgen administration based on two-dimensional isotopic fingerprinting against an endogenous internal standard"; £116,000 for 1.5 years.
- Co-Investigator on Dr Michael Richards' (University of Bradford) NERC research proposal (EFCHED programme): "The evolution of hominid dietary adaptations linked with environmental changes: extending the record beyond 100,000 years"; £123,220 for 3 years.
- Wellcome Trust Project Grant jointly with Prof. Michael J Rennie (PI) (School of Life Sciences, Dundee) to investigate "New approaches to the study of human collagen metabolism in normal adults and after burn injury in children"; July 2000 to June 2002, £360,841.
- Research grant (PI) in collaboration with Dr John F Dillon (Gastroenterology & Hepatology, Ninewells Hospital and Medical School, Dundee) and Dr Anne Rühl (Gastroenterology, University's Hospital Trust, Heidelberg, Germany) to develop "Stable Isotope Labelled Probes for Non-Invasive Assessment of Physiological Functions in the Human Body"; awarded by the Dietmar-Hopp-Stiftung für Medizinische Forschung und Studien (Germany), February 1999 to January 2002, £356,411.
- Research contract (PI) awarded by Phenome Sciences Inc, MassTrace Group, Woburn, MA (USA), for work on new stable isotope labelled probes to assess gastric emptying by means of a $^{13}\text{CO}_2$ -breath test; January 2001, £10,240.
- Industrial grant (PI) awarded by Europa Scientific Ltd., Crewe, for work on GC/C-IRMS application and instrument development; October 1997 to September 1998, £113,985.

Recent unsuccessful Grants / Research Proposals:

- NERC small grant application: "Linking Dietary and Environmentally Derived Signals in Human Tissues to Life Chronology of Modern Man"; as PI together with Prof SM Black (University of Dundee).
- BBSRC standard grant application: "Linking Isotopic Signals to Geographical Origin of Living People and Human Remains"; as PI together with Profs SM Black (University of Dundee) and MP Richards (University of Bradford); invited for resubmission to NERC as archaeologically focused proposal.

Membership of EPSRC funded Networks

- ❖ Forensic Isotope Ratio Mass Spectrometry Network (FIRMS)
- ❖ International Crime Science Network
- ❖ Geoforensics and Information Management for crime Investigation (GIMI).

Collaborations:

- With Dr Niamh NicDaeid (CFS, University of Strathclyde): "Isotope Profiling of Drugs to Combat Serious and Organised Crime" and "Multi-dimensional Profiling of Drugs and Fibres".
- With Dr Damian McKay (Royal Victoria Hospital, Queen's University Belfast): "Monitoring Ulcerative Colitis and its therapy by means of a non-invasive breath test".
- With Prof Sue Black on the identification and determination of geographic origin of archaeological human remains (Seaman Swan).
- With Prof Kenneth Leung and Prof David Dudgeon (Dept. of Ecology & Biodiversity, The University of Hong Kong): "Are stable carbon isotope ratios of essential and non-essential fatty acids accurate tracers in studies of aquatic food webs?"
- With Dr Lesley Houghton (Neurogastroenterology Unit, Academic Division of Medicine and Surgery, University of Manchester): "Can hypnosis be used to induce nausea and is this associated with delayed gastric emptying?"

Forensic Case Work

- Registered forensic expert advisor with the UK's National Crime & Operations Faculty (now National Centre for Police Excellence).
- An Garda Síochána (Dublin): Murder investigation.
- Durham Constabulary: Unidentified body.
- Lothian & Borders Police: Abandoned baby ('Baby Gary').
- National Crime & Operations Faculty: Murder investigation.
- Police Service Northern Ireland: Two murder investigations and one missing persons (presumed to be murdered).
- Strathclyde Police: One case of unidentified skeletal remains (missing persons / possible foul play).

Consultancy Services

- Consultancy services for Adria Ltd, Strabane, Co. Tyrone; July 2003 – April 2004; £18,964.
- Contract study for Guinness-UDV, Brand Innovation Group; May – October 2002; £83,255.
- Consultancy services for Guinness-UDV, Brand Innovation Group, Bishops Stortford, Hertfordshire; July 2001.
- Consultancy services for Isotope Analytical Services Ltd, Bridge of Don, Aberdeen, UK; May 2001.
- Co-founded ASSIST (Applied Scottish Stable Isotope Technology) as commercial arm of Dundee's stable isotope facility in March 2000. Total value of contract analyses: £4,778.
- Consultancy services for Ricardo Consulting Engineers, Shoreham-on-Sea, West Sussex, UK; January 1999.

Membership of Professional Bodies

- Member of the Royal Society of Chemistry and Chartered Chemist.
- Member of the British Mass Spectrometry Society.
- Member of the UK Alexander-von-Humboldt Association.

Outside Recognition:

- External PhD examiner, University of Oxford (UK), 2004.
- External PhD examiner, Swedish University of Agricultural Sciences, Uppsala / Umea, 2005.
- Key-note presentation at the 4th European Academy of Forensic Sciences Conference (EAFS, 13-16 June 2006, Helsinki).
- Programme chair at the 4th the European Academy of Forensic Sciences Conference (EAFS, 13-16 June 2006, Helsinki).
- Invited contributor to a five day course on "Principles and Practice of Stable Light Isotopes" at the University of Bradford (3 – 7 July 2006).
- Member of programme committee of the 5th international conference on application of isotope techniques to ecological studies (ISOECOL, 13-18 August 2006, Belfast).
- Session chair at the 5th international conference on application of isotope techniques to ecological studies (ISOECOL, 13-18 August 2006, Belfast).

Patents and Patent Applications:

1. W. MEIER-AUGENSTEIN: "Gas Inlet System", *Int. Patent Appl. No. PCT/GB96/03213*; *British Patent Appl. No. 9526508.8*; *Publication No. WO 97/23770*.
2. W. MEIER-AUGENSTEIN, J.A. Cairns and J. Thomson: "Catalytic Conversion Interface", *Int. Patent Appl. No. PCT/GB02/00172*; *British Patent Appl. No. GB0101164.2*; *Publication No. WO 02/057007 A2*.
3. W. MEIER-AUGENSTEIN: "Gastric Emptying Test", *Int. Patent Appl. No. PCT/GB02/00528*; *British Patent Appl. No. GB103097.2*; *Publication No. WO 02/062399 A1*.
4. W. MEIER-AUGENSTEIN: "Liver Function Test", *Int. Patent Appl. No. PCT/GB02/01310*; *British Patent Appl. No. GB106923.6*; *Publication No. WO 02/075320 A2*.
5. J. Thomson and W. MEIER-AUGENSTEIN: "CO₂ / NO_x Sensor", *Int. Patent Appl. No. PCT/GB02/03888*; *British Patent Appl. No. GB0120962.6*; *Publication No. WO 03/021245 A2*.
6. W. MEIER-AUGENSTEIN: "Method of Fabric Odour Detection"; *Int. Patent Appl. No. PCT/GB2004/00145*.
7. W. MEIER-AUGENSTEIN: "Breath Test Phenotyping for Adverse Drug Reactions"; *British Patent Appl. No. GB0621829.1*.

John Kenneth Amory MD, MPH, FACP

Office: University of Washington, Box 356429
1959 NE Pacific St
Seattle, WA 98195
Tel: (206) 616-1727 fax: (206) 616-5365
Email: jamory@u.washington.edu
<http://depts.washington.edu/gim/faculty/amory.htm>

Home: 1227 17th Ave E
Seattle, WA 98112-3316
(206) 324-1784

PERSONAL INFORMATION

Born: April 19, 1967, Vancouver, British Columbia, Canada (US citizen)

Married: October 7, 1995 to Josephine Harris Amory MD (Obstetrics)

Children: William Glendinning Amory, born July 5th, 2002

Thomas Gerard Amory born Feb 1st, 2005

EDUCATION

2004-2006 MPH, University of Washington, Seattle, WA

1989-1994 MD with Thesis, University of California, San Francisco, CA

1985-1989 BA Biology-*Magna cum Laude*, Harvard University, Cambridge, MA

POSTGRADUATE TRAINING

1994-1997 Resident in Internal Medicine, University of California, San Francisco, CA

1992-1993 Research Fellow, National Institutes of Health, Bethesda, MD

FACULTY POSITIONS

2005- Associate Professor of Medicine, University of Washington, Seattle, WA

2001-2005 Assistant Professor of Medicine, University of Washington, Seattle, WA

1997-2001 Acting Instructor of Medicine, University of Washington, Seattle, WA

HOSPITAL POSITIONS

2001- Attending Physician, University of Washington, Seattle, WA

1997-2001 Staff Physician and Director Pre-op clinic, VA-Puget Sound, Seattle, WA

HONORS & AWARDS

| | |
|------|---|
| 2007 | Young Andrologist Award, American Society of Andrology |
| 2006 | Named one of "Seattle's Best Doctors" by <i>Seattle Magazine</i> (Internal Med) |
| 2005 | Endocrine Society International Award for Excellence in Published Clinical Research |
| 2004 | Helen and Phillip Fialkow Scholar, Department of Medicine, University of Washington |
| 2003 | Paul Beeson Award for excellence in Housestaff Instruction, Department of Medicine, University of Washington |
| 2003 | AOA honorary inductee, University of Washington Medical School graduating class of 2003 |
| 2003 | Fellowship, American College of Physicians |
| 1997 | Weingarten Memorial Award for outstanding third-year medicine resident, University of California, San Francisco |
| 1996 | Martin Memorial Award for outstanding second-year medicine resident, University of California, San Francisco |
| 1992 | Dean's Prize for Medical Student Research, UCSF |
| 1990 | Dr. June Colburn Research Fellow, UCSF |
| 1987 | John Harvard Scholar |
| 1985 | National Merit Scholar |

CERTIFICATIONS & LICENSURE

| | |
|--------------|---|
| 1997-present | American Board of Internal Medicine, Board Certified in Internal Medicine (renewed 2007-2017) |
| 1997-present | Washington Medical License |
| 1996-present | DEA License |

PROFESSIONAL ORGANIZATIONS

| | |
|--------------|---|
| 1999-present | The Andrology Society |
| 1997-present | American College of Physicians |
| 2003-present | The Endocrine Society |
| 2003-present | Association of Reproductive Health Professionals (Planned Parenthood) |

TEACHING RESPONSIBILITIES

YEARLY:

1. Six weeks ward attending in general inpatient medicine. Responsible for teaching rounds and overseeing patient management.
2. Multiple lectures to residents on general medicine topics including thyroid disease, nutrition, contraception, reproductive disorders, medication side effects and peripheral vascular disease.
3. Human Biology 565 (2nd year medical student course on human reproduction). Co-course director (with Robert Steiner, PhD). 5 hours of course lectures yearly: "The

Adult Male," "Male Infertility," "Male Contraception," "Population Dynamics,"
"Androgens in the Aging Male."

4. Human Biology 665 (3rd year medical student medical clerkship lecture series). 4 hours of course lectures yearly (Acute Renal Failure)
5. Human Biology 544 (2nd year medical student course on endocrinology). Small group leader "Hypothyroidism" "Adrenal Insufficiency"

EDITORIAL RESPONSIBILITIES

At-large reviewer for *Journal of Clinical Endocrinology and Metabolism*, *Endocrinology*, *Human Reproduction*, *Journal of Andrology*, *Asian Journal of Andrology* and others

NATIONAL RESPONSIBILITIES

| | |
|--------------|--|
| 2004-present | United States Anti-Doping Agency, anti-doping review board |
| 2005-present | Reviewer, Faculty of 1000, Reproductive Physiology |

UNIVERSITY SERVICE

| | |
|--------------|---|
| 2004-present | Medical School Admissions Committee |
| 2004-present | Human Subjects (IRB) Committee A-member |
| 2003 | Residency Selection Committee |
| 2002-2004 | Faculty Senate-member |
| 2001-present | UW Hospital Nutrition Committee (Chair) |

CURRENT RESEARCH FUNDING

1. NIH/NICHD
1K23HD045386, 9/04-9/09
PI: John K. Amory Total costs: \$629,668
Oral Androgen Administration and Function in Man
2. NIH/NICHD
Contraceptive Clinical Trials Network
PI: William J. Bremner
John K. Amory, Co-investigator
Contract #: HHSN27520040337
Safety and Gonadotropin Suppressive Activity of Nestorone Gel and Testosterone Gel in Men 4/05-4/07
Total costs: \$225,866
3. NIH/NICHD
U54 HD42454
Center PI: William J. Bremner 9/02-9/07
Project I PI: William J. Bremner
John K. Amory, Co-investigator
Project I: Hormonal Contraception Total costs: \$9.5 m
In the Male Project I costs:
4. NIH/NICHD

- U54 HD012629-27
Center P.I: Robert Braun
Project V PI: William J. Bremner
John K. Amory, Co-investigator
Project V: Gonadotropin Regulation
Steroidogenesis, spermatogenesis and gene
Expression in man
- 4/06-3/11
Total costs: \$10.6 m
Project V costs: 1.5m
5. University of Washington, Center for Research in
Reproduction and Contraception
PI: John K. Amory
Contraceptive pilot grant: Gonadotropin suppression
with oral testosterone enanthate and finasteride
in man
- 6/06-5/07
Total costs: \$35,000
6. NIH/NICHD
HD-05-040
PI: William J. Bremner
Research Mentor/Program Director: **John K. Amory**
Men's Reproductive Health Research
at the University of Washington/
K12 Training program
- 9/06-9/11
Total costs: 2.2 m

PENDING RESEARCH FUNDING

1. GlaxoSmithKline
TDC106220
PI: John K. Amory
Oral nanomilled testosterone in hypogonadal men
- Dates: 1/07-1/08
Total costs: \$310,821
2. Merriam Pharmaceuticals
PI: John K. Amory
Oral administration of the GnRH antagonist acyline
In normal men
- Dates: 3/07-3/08
Total costs: \$100,000
3. NIH/NICHD
RFA-HD-06-014
Center PI: William J. Bremner
Project I PI: **John K. Amory**
Oral testosterone for male hormonal contraception
- 3/07-2/12
Total costs: 13.5m
Project I costs: 2.2m

COMPLETED RESEARCH FUNDING

1. American Professors of Infection Control.
P.I. John K. Amory
Computer-based prevention of urinary tract infection
- 1/00-1/01
Total costs: \$8,000
2. University of Washington, Center for Research in

- | | |
|--|-----------------------|
| Reproduction and Contraception | 5/04-5/06 |
| PI: John K. Amory | Total costs: \$68,211 |
| Contraceptive Pilot Grant: Glycosphingolipid Inhibition and Spermatogenesis in Men | |
-
- | | |
|---|------------------------|
| 3. Schering Pharmaceuticals | 3/05-8/06 |
| PI: John K. Amory | Total costs: \$150,000 |
| Glycosphingolipid Inhibition and Spermatogenesis in Men | |
-
- | | |
|--------------------------|------------------------|
| 4. GlaxoSmithKline, | 12/04-12/06 |
| PI: John K. Amory | Total costs: \$169,000 |
| Oral Androgens in Man | |

PUBLICATIONS

PEER-REVIEWED ARTICLES

1. **Amory JK**, Chou T, Redberg R, Blake L, Vartanian R. Diagnosis of a Primary Cardiac Leiomyosarcoma by Endomyocardial Biopsy. *Cardiovascular Pathology* 1996, Vol 5(2): 113-117.
2. Eisner M, **Amory JK**, Mullaney B, Tierney L Jr., Browner WS. Necrotizing lymphadenitis associated with systemic lupus erythematosus. *Seminars in Arthritis and Rheumatism* 1996, 26(1): 477-482.
3. **Amory JK**, Matsumoto AM. The therapeutic potential of testosterone patches. *Expert Opinion in Investigational Drugs* 1998 7(12): 1977-1985.
4. **Amory JK**, Bremner WJ. The Use of Testosterone as a Male Contraceptive. *Balliere's Clinics in Endocrinology* 1998 12(3): 471-483.
5. **Amory JK**, Bremner WJ. Newer agents for hormonal contraception in the male. *Trends in Endocrinology and Metabolism* 2000, 11(2): 61-66.
6. **Amory JK**, Anawalt BD, Paulsen CA, Bremner WJ. Klinefelter syndrome: a brief review with a biography of Dr. Klinefelter. *Lancet* 2000, 356:333-335.
7. Kahn JG, Becker BJ, MacIssac L, **Amory JK**, Neuhaus J, Olkin I, Creinin M. The efficacy of medical abortion: A meta-analysis. *Contraception* 2000, 61:29-40.
8. Saint S, Wiese J, **Amory JK**, Bernstein ML, Patel UD, Zemencuk JK, Bernstein SJ, Lipsky BA, Hofer TP. Are physicians aware of which of their patients have an indwelling urinary catheter? *American Journal of Medicine* 2000, 109:476-480.
9. **Amory JK**, Bremner WJ. Endocrine regulation of testicular function in men. *Molecular and Cellular Endocrinology* 2001, 182:175-179.
10. Goldstein AS, **Amory JK**, Martin SM, Vernon C, Matsumoto AM, Yager P. Testosterone delivery using glutamide based complex high axial ratio microstructures. *Bioorganic and Medicinal Chemistry* 2001, 9:2819-2825.
11. **Amory JK**, Anawalt BD, Bremner WJ, Matsumoto AM. Daily testosterone and gonadotropin levels are similar in azoospermic and nonazoospermic normal men administered weekly testosterone: implications for male contraceptive development. *Journal of Andrology* 2001, 22:1053-1060.

12. Anawalt BD, **Amory JK**. Male hormonal contraceptives. *Expert Opinion in Pharmacotherapeutics* 2001, 2:1389-1398.
13. Anawalt BD, **Amory JK**. Male contraception. *Annals of Medicine* 2001; 587-595.
14. **Amory JK**, Anawalt BD, Blaskovich PD, Gilchrist J, Nuwayser ES, Matsumoto AM. Testosterone release from a subcutaneous, biodegradable microcapsule formulation (Viatrel) in hypogonadal men. *Journal of Andrology* 2002, 23:84-91.
15. Amory DW, Grigore A, **Amory JK**, Gerhardt MA, White WD, Smith PK, Schwinn DA, Reves JG, Newman MF. Neuroprotection is associated with beta-adrenergic receptor antagonists during cardiac surgery: Evidence from 2,575 patients. *Journal of Cardiothoracic and Vascular Anesthesia* 2002, 16:270-277.
16. Cherrier MM, Anawalt BD, Herbst KL, **Amory JK**, Craft S, Matsumoto AM, Bremner WJ. Cognitive effects of short-term manipulation of serum sex steroids in healthy young men. *Journal of Clinical Endocrinology and Metabolism* 2002, 87:3090-3096.
17. Herbst KL, Anawalt BD, **Amory JK**, Bremner WJ. Acyline: the first study in humans of a potent, new gonadotropin-releasing hormone antagonist. *Journal of Clinical Endocrinology and Metabolism* 2002, 87:3215-3220.
18. **Amory JK**, Chansky HA, Chansky KL, Camuso M, Hoey C, Anawalt BD, Matsumoto AM, Bremner WJ. Preoperative supraphysiological testosterone in older men undergoing knee replacement surgery. *Journal of American Geriatrics Society* 2002, 50:1698-1701.
19. Herbst KL, Anawalt BD, **Amory JK**, Matsumoto AM, Bremner WJ. The male contraceptive regimen of testosterone and levonorgestrel significantly increases lean mass in healthy young men in 4 weeks but attenuates a decrease in fat mass induced by testosterone alone. *Journal of Clinical Endocrinology and Metabolism* 2003,88:1167-1173.
20. **Amory JK**. Male Contraception. *A.R.T. and Science* 2003; 2(4):8-11.
21. Cornia P, **Amory JK**, Fraser S, Saint S, Lipsky B. Computer-based order entry decreases duration of indwelling urinary catheterization in hospitalized patients. *American Journal of Medicine* 2003, 114:404-407.
22. **Amory JK**, Bremner WJ. Regulation of testicular function in experimental male contraceptive development. *Journal of Steroid Biochemistry and Molecular Biology* 2003,86:357-361.
23. Herbst KL, **Amory JK**, Brunzell JD, Chansky HA, Bremner WJ. Testosterone administration to men increases hepatic lipase activity and decreases HDL and LDL size in 3 weeks. *American Journal of Physiology: Endocrinology and Metabolism* 2003, 284:E1112-E1118.
24. **Amory JK**, Scriba GKE, Amory DW, Bremner WJ. Oral testosterone-triglyceride conjugate in rabbits: Single-dose pharmacokinetics and comparison with oral testosterone undecanoate. *Journal of Andrology* 2003, 24:716-720.
25. **Amory JK**. George Washington's infertility: why the father of our country was never a father. *Fertility and Sterility* 2004; 81:495-499.

26. Gui YL, He CH, **Amory JK**, Bremner WJ, Zheng EX, Yang J, Yang PJ, Gao ES. Male Hormonal Contraception: Suppression of spermatogenesis by injectable testosterone undecanoate alone or with levonorgestrel implants in Chinese men *Journal of Andrology*, 2004 25:720-727.
27. ***Amory JK**, Watts NB, Easley KA, Sutton PR, Anawalt BD, Matsumoto AM, Bremner WJ, Tenover JL. Exogenous testosterone or testosterone with finasteride increases bone mineral density in older men with low serum testosterone. *Journal of Clinical Endocrinology and Metabolism* 2004; 89:503-510.
28. Coviello AD, Bremner WJ, Matsumoto AM, Herbst KL, **Amory JK**, Anawalt BD, Yan X, Brown T, Wright WW, Zirkin BR, Jarow JP. Intratesticular testosterone concentrations levels comparable to serum levels are not sufficient to maintain normal sperm production spermatogenesis in men receiving a hormonal contraceptive regimen. *Journal of Andrology*, 2004 25:931-938.
29. **Amory JK**. The testosterone/progestin pill: A realistic option for male contraception? *Current Opinion in Investigational Drugs* 2004 5:1025-1030.
30. Herbst KL, Coviello AD, Page ST, **Amory JK**, Anawalt BD, Bremner WJ. Acyline, a gonadotropin releasing-hormone antagonist suppresses gonadotropins and testosterone for 15 days after a single dose. *Journal of Clinical Endocrinology and Metabolism*, 2004 89:5959-5965.
31. Matthiesson KL, **Amory JK**, Berger R, Ugoni A, McLachlan RI, Bremner WJ. Novel male hormonal contraceptive combinations: The hormonal and spermatogenic effects of testosterone and levonorgestrel combined with a 5-alpha-reductase inhibitor or GnRH Antagonist. *Journal of Clinical Endocrinology and Metabolism*, 2005 90:91-97.
32. Page ST, Herbst KL, **Amory JK**, Coviello AD, Matsumoto AM, Bremner WJ. Testosterone suppresses adiponectin levels in men. *Journal of Andrology*, 2005 26:85-92.
33. Cherrier MM, Matsumoto AM, **Amory JK**, Ahmed S, Bremner WJ, Peskind E, Raskind MA, Johnson M, Craft S. The role of aromatization in testosterone supplementation: Effects on cognition in older men. *Neurology* 2005; 64:290-296.
34. Page ST, **Amory JK**, Bowman ED, Anawalt BD, Matsumoto AM, Bremner WJ, Tenover JL. Exogenous Testosterone (T) Alone or with Finasteride Increases Physical Performance, Grip Strength, and Lean Body Mass in Older Men with Low Serum T. *Journal of Clinical Endocrinology and Metabolism*, 2005: 1502-1510.
35. Anawalt BD, **Amory JK**, Herbst KL, Coviello AD, Bremner WJ, Matsumoto AM. Very low-dosage oral levonorgestrel plus intramuscular testosterone enanthate suppresses spermatogenesis without causing weight gain in normal young men: a randomized clinical trial. *Journal of Andrology*, 2005 26:405-413.
36. ***Amory JK**, Bremner WJ. Oral testosterone in oil plus dutasteride: a pharmacokinetic study in men. *Journal of Clinical Endocrinology and Metabolism*, 2005; 90:2610-2617.
37. Coviello AD, Matsumoto AM, Bremner WJ, Herbst KL, **Amory JK**, Anawalt BD, Sutton PL, Wright WW, Brown T, Yan X, Zirkin BR, Jarow JP. Low dose human chorionic gonadotropin maintains intratesticular testosterone in normal men

- with testosterone induced gonadotropin suppression. *Journal of Clinical Endocrinology and Metabolism*, 2005; 90:2595-2602.
38. Cherrier, MM, Matsumoto AM, **Amory JK**, Asthana S, Bremner, WJ, Peskind ER, Raskind MA, Craft S. Testosterone Improves Spatial Memory in Men with Alzheimer's Disease and Mild Cognitive Impairment. *Neurology*, 2005; 64:2063-2068.
 39. Matthiesson KL, Stanton PG, O'Donnell L, Meachem S, **Amory JK**, Berger R, Bremner WJ, McLachlan RI. Effects of testosterone and levonorgestrel combined with a 5alpha reductase inhibitor or GnRH antagonist on spermatogenesis and intratesticular steroid levels in normal men. *Journal of Clinical Endocrinology and Metabolism* 2005; 90:5647-5655.
 40. **Amory JK**. Male Hormonal Contraception: Current status and future prospects *Treatments in Endocrinology* 2005; 4(6):333-41.
 41. Brady DM, **Amory JK**, Perheentupa A, Zitzmann M, Hay C, Apter D, Anderson RA, Bremner WJ, Huhtaniemi I, Nieschlag E, Wu FCW, Kersemaekers WM. A multi-centre study investigating subcutaneous etonogestrel implants with injectable testosterone decanoate as a potential long-acting male contraceptive. *Human Reproduction*, 2006;21:285-294.
 42. ***Amory JK**, Page ST, Bremner WJ. Oral testosterone in oil: Pharmacokinetic effects of 5 α reduction with finasteride or dutasteride and food intake in men. *Journal of Andrology* 2006;27:72-78.
 43. **Amory JK**, Page ST, Bremner WJ. Recent progress in male hormonal contraception. *Nature Clinical Practice: Endocrinology and Metabolism* 2006;2:32-41.
 44. Page ST, Plymate SR, Bremner WJ, Hess DL, Matsumoto AM, Lin DW, **Amory JK**, Nelson PS, Wu JD. Effects of medical castration and testosterone replacement on CD4+CD25+ regulatory T cells, CD8+ T-cell IFN γ expression and NK cells: Evidence for a physiological role for testosterone and/or its metabolites in cellular immune function *American Journal of Physiology: Endocrine and Metabolism* 2006 290(5):E856-63
 45. **Amory JK**, Bremner WJ. Male hormonal contraception: the future of male contraception? *Medicine* 2006 34:1 25-26.
 46. **Amory JK**, Rosen H, Sukit C, Wallace F, Saint S. A Jaundiced Eye: Clinical Problem Solving. *New England Journal of Medicine* 2006 324;14:62-66.
 47. **Amory JK**. Male hormonal contraceptives. *Minerva Ginecol.* 2006 58:215-26.
 48. Page ST, Lin DW, Mostaghel EA, Hess DL, True LD, **Amory JK**, Nelson PS, Matsumoto AM, Bremner WJ. Persistent intraprostatic androgen concentrations after medical castration in healthy men. *Journal of Clinical Endocrinology and Metabolism* 2006 91:3850-3856.
 49. Page ST, **Amory JK**, Anawalt BD, Irwig M, Brockenbrough A, Matsumoto AM, Bremner WJ. Testosterone gel combined with depomedroxyprogesterone acetate (DMPA) is an effective male hormonal contraceptive regimen but is not enhanced by the addition of the GnRH antagonist acyline. *Journal of Clinical Endocrinology and Metabolism* 2006 91:4374-4380.

50. Kalus R, Shojania KG, **Amory JK**, Saint S. Lost in Transcription: Clinical Problem Solving. *New England Journal of Medicine* 2006 355:1487-1491.
51. ***Amory JK**, Page ST, Anawalt BD, Matsumoto AM, Bremner WJ. Acceptability of a combination testosterone gel and depomedroxyprogesterone acetate male contraceptive regimen. *Contraception* 2007 75:218-223.
52. **Amory JK**, Muller CH, Page ST, Pagel ER, Bhandari A, Leifke E, Subramanyam B, Bone W, Radlmier A, Bremner WJ. Miglustat has no apparent effect on spermatogenesis in normal men. *Human Reproduction* 2007 22:702-707.
53. Cherrier, MM, Matsumoto AM, **Amory JK**, Johnson M, Holman G, Craft S, Peskind ER, Raskind MA. Characterization of verbal and spatial memory changes from moderate to supraphysiological increases in serum testosterone in healthy older men. *Psychoneuroendocrinology* 2007 32:72-79.
54. ***Amory JK**, Wang C, Swerdloff R, Anawalt BD, Matsumoto AM, Bremner WJ, Walker SE, Haberer LJ, Clark RV. The Effect of 5 α -Reductase Inhibition with Dutasteride and Finasteride on Semen Parameters and Serum Hormones in Healthy Men. *Journal of Clinical Endocrinology and Metabolism* Epub 2/13/07.
55. **Amory JK**, Page ST, Anawalt BD, Coviello AD, Matsumoto AM, Bremner WJ. Elevated end-of-treatment serum INSL3 is associated with failure to completely suppress spermatogenesis in men receiving male hormonal contraception. *Journal of Andrology* Epub 2/21/07.
56. **Amory JK**. Contraceptive developments for men. *Drugs of Today* 2007 43(3): 179-192.
57. **Amory JK**, Coviello AD, Page ST, Anawalt BD, Bremner WJ. Serum 17-hydroxyprogesterone strongly correlates with intratesticular testosterone in gonadotropin-suppressed normal men receiving various dosages of human chorionic gonadotropin *Fertility and Sterility* (in press).

BOOK CHAPTERS

58. **Amory JK**. "Common problems of the elbow." In: Frances C, Bent S and Saint S Eds. *Saint-Francis Guide to Outpatient Medicine*. Lippincott Williams and Wilkins, Philadelphia 1999:365-368.
59. **Amory JK**. "An approach to injection and aspiration of joints." In: Frances C, Bent S and Saint S Eds. *Saint-Francis Guide to Outpatient Medicine*. Lippincott Williams and Wilkins, Philadelphia, 1999:335-339.
60. **Amory JK**, Bremner WJ. "Male contraception" In *Principles and Practice of Endocrinology and Metabolism* (3rd Ed.) Becker KL (Ed.) Lippincott Williams and Wilkins, Philadelphia 2001: 1220-1224.
61. Bremner WJ, Huhtaniemi I, **Amory JK**. "Pituitary gonadotropins and their disorders" In *Principles and Practice of Endocrinology and Metabolism* (3rd Ed.) Becker KL (Ed.) Lippincott Williams and Wilkins, Philadelphia 2001:170-177.
62. Rooke A, **Amory JK**. "Anesthesia and perioperative management of the geriatric patient." In *Orthopedics*, Fitzgerald R, Kaufer H, Malkani A (Eds.) Mosby, Philadelphia 2002:20-29.

63. **Amory JK**, Bremner WJ. "New Developments in Male Contraception". In *Gynecology and Obstetrics*. Lippincott, Williams, Wilkins, 2003, Vol. 6, chapter 13 (CD-ROM Textbook).
64. **Amory JK**. "Male Hormonal Contraception". In. *Androgens in Health and Disease*. Eds. Carrie Bagatell and William Bremner. Humana press, Totowa, New Jersey 2003:405-418.
65. Sutton PR, **Amory JK**, Clark R. "DHT and 5 α reductase: normal physiology and inhibition". In. *Androgens in Health and Disease*. Eds. Carrie Bagatell and William Bremner, Humana Press, Totowa, New Jersey 2003:77-88.
66. **Amory JK**. "Klinefelter syndrome" in *Male Hypogonadism: Basic, Clinical and Therapeutic Principles*. Ed. Stephen Winters. Humana press, Totowa, New Jersey 2003:157-167.
67. **Amory JK**. "Male Contraception" in *Current Topics in Andrology*. Ed. Phillip Matson, Ladybrook Press, West Leederville, Western Australia, 2003:161-182.
68. Matsumoto AM, **Amory, J.K.** Effects of aging: fertility, menopause and changes in men. In: *Endocrinology of Fertility and the Reproductive System* D. Spratt, N. Santoro, eds, Humana Press, 2004:240-257.
69. **Amory JK**, Bremner WJ. Therapeutic efficacy of androgen delivery systems. In: *Updates in Infertility Treatment*. M Filcore, Ed. Medimond, Bologna, Italy, 2004:333-350.
70. **Amory JK**. "Klinefelter Syndrome" in *The Encyclopedia of Human Development*. Salkind, Neil J. (Ed.). Sage Publications, Thousand Oaks, CA, 2005.
71. **Amory JK**. "Adult diabetic ketoacidosis" in *Manual of Evidence-Based Admitting Orders and Therapeutics* (5th Ed.). Eds. Eric B. Larson and Karen McDonough, Elsevier, London, 2006: 43-47.
72. **Amory JK**. "Hyperosmolar hyperglycemic state and hyperosmolar nonketotic coma" in *Manual of Evidence-Based Admitting Orders and Therapeutics* (5th Ed.). Eds. Eric B. Larson and Karen McDonough, Elsevier, London, 2006: 48-53.

OTHER PUBLICATIONS

73. **Amory JK**: Preparation, titer, affinity and use of immunoglobulin isolated from eggs of immunized hens. (Undergraduate Thesis) Harvard College, 1989.
74. **Amory JK**: The role of Pr60^{gag} in the immunopathogenesis of Murine AIDS. (MD Thesis) University of California, San Francisco, 1994.
75. **Amory JK**. Cancer therapy and sperm banking (letter to the editor). *New England Journal of Medicine*, 2004 351:510.
76. **Amory JK**, Amory DW. Oral erythromycin and the risk of sudden death (letter to the editor). *New England Journal of Medicine* 2005 352:301-304.
77. Clark B, **Amory JK**. Research in male contraception. *The Female Patient*, 2005; 30:28-31.
78. Arias E, **Amory JK**. Testosterone and the Male Heart: Friend or Foe? *Renal and Urology News* 2006 1(4):1-8.
79. **Amory JK**, Amory DW. Dosing frequency of aspirin and prevention of heart attack and strokes (letter to the editor). *American Journal of Medicine* 2007 (in press).

CURRICULAR PUBLICATIONS

1. **Amory JK.** Human Biology 565 syllabus: "The Adult Male," "Male Infertility," "Male Contraception," "Population Dynamics," "Androgens in the Aging Male." (yearly)
2. Bussell S, DeHoog S, Billingsly S, **Amory JK.** "Hospital Nutrition for 3rd year medical students." <http://courses.washington.edu/med665/nutrition/>

PATENTS

1. "Oral Androgen Therapy Using Modulators of Testosterone Bioavailability" US Patent # 10,990,118 John K. Amory and William J. Bremner (Holder University of Washington)- issued 11/21/06.

MANUSCRIPTS SUBMITTED

1. Matthiesson KL, Meachem SJ, **Amory JK**, Robertson DM, Bremner WJ, McLachlan RI. Relationship of serum INSL3 to germ cell and Leydig cell parameters in normal men receiving male hormonal contraceptive treatment. (Submitted to *Journal of Clinical Endocrinology and Metabolism*).
2. Page ST, Bremner WJ, Clark RV, Bush MA, Carifcofe RB, Smith PM, **Amory JK.** Oral nanomilled testosterone plus dutasteride effectively normalizes serum testosterone in medically castrate men. (Submitted to *Journal of Clinical Endocrinology and Metabolism* 2/07)
3. Page ST, Kalhorn T, Bremner WJ, Anawalt BD, Matsumoto AM, **Amory JK.** Intratesticular androgens and spermatogenesis during male hormonal contraceptive treatment (Submitted to *Journal of Clinical Endocrinology and Metabolism* 1/07)
4. Kalus A, Fredericks LP, Presland R, Livingston B, **Amory JK**, Sonesson A, Back O, Dale B. Human-B-defensin 1 polymorphism is associated with allergic sensitization in atopic dermatitis. (Submitted to *The Lancet*)

ABSTRACTS

1. **Amory J**, Kliks S, Levy J: Effect of Anti-HIV Monoclonal Antibodies on HIV-1 Replication *in vitro*. Clinical Research, 1991 39:58A.
2. **Amory J**, Martin N, Levy J, and Wara D: The Large Molecular Weight Glycoprotein MG1, A Component of Human Saliva Inhibits HIV-1 Infectivity. Clinical Research, 1992 40:51A.
3. Anawalt BD, **Amory JK**, Herbst KL, Matsumoto AM, Bremner WJ. Testosterone administration to normal men decreases truncal and total body fat. Clinical Research, 1999
4. **Amory JK**, Chanksy H, Chansky K, Hoey C, Anawalt BD, Matsumoto AM and Bremner WJ. The effects of supraphysiologic testosterone on functional outcomes after joint replacement surgery in elderly men. ENDO 99, OR 9-3 (oral presentation).
5. **Amory JK**, Anawalt BD, Matsumoto AM. Safety and pharmacokinetics of testosterone release from an injected microcapsule in hypogonadal men. American Society of Andrology, 2000.

6. **Amory JK**, Bremner WJ, Herbst KL, Matsumoto AM, Anawalt BD. Testosterone rapidly increases vertebral bone mineral density in eugonadal men. ENDO 2000, Abstract #852276.
7. Herbst KL, Anawalt BD, **Amory JK**, Matsumoto AM, Bremner WJ. Exogenous testosterone administration to normal men changes body composition from fat to lean without changing body weight. ENDO 2000, Abstract #2354.
8. Dick SE, Anawalt BD, **Amory JK**, Herbst KL, Bremner WJ, Matsumoto AM, Lower dose levonorgestrel plus testosterone enanthate effectively suppresses spermatogenesis with little or no weight gain. ENDO 2000, abstract #2353.
9. Herbst KL, Deeb SS, Bremner WJ, **Amory JK**. Testosterone increases hepatic lipase and decreases HDL-C in three weeks in elderly men. *W Soc Clin Investigation* 2002.
10. Anawalt BD, **Amory JK**, Wang C, Swerdloff RS, Dobs AS, Meikle AW, Elbers JMH, Houwing NS. A pharmacokinetic study of oral testosterone undecanoate. *Andrology Society* 2002.
11. Herbst KL, Anawalt BD, Coviello A, **Amory JK**, Bremner WJ. Acyline: a New potent, long-acting Gonadotropin-releasing hormone (GnRH) antagonist safely and significantly suppresses testosterone for greater than two weeks after a single dose. Endocrine Society 2002, Abstract # P2-654.
12. Herbst KL, **Amory JK**, Chansky HA, Bremner WJ. Supraphysiological testosterone administration rapidly increases hepatic lipase activity (HLA) and decreases HDL(2) and HDL (3) and LDL size in older men. Endocrine Society 2002, Abstract #P2-653.
13. Coviello A, **Amory JK**, Anawalt BD, Matsumoto AM, Bremner WJ. Gonadotropins are higher in men who fail to suppress to azoospermia compared to those who do not. Endocrine Society 2002, Abstract #
14. Voie A, Amory D, **Amory JK**, Moehring M, Spencer M. Ultrasound and tPA enhanced thrombolysis: influence of ultrasound frequency and pulse length. European Society of Neurosonology and Cerebral Hemodynamics, 2002, P157
15. Coviello AD, Herbst KL, **Amory J**, Anawalt B, Jarow JP, Brown T, Wright B, Bremner W, Matsumoto AM. Intratesticular testosterone is 40 fold higher than serum testosterone in normal men but is successfully suppressed by testosterone enanthate and levonorgestrel. *W Soc Clin Investigation*, 2003
16. Herbst KL, Coviello AD, **Amory JK**, Anawalt BD, Murdoch SJ, Brunzell JD, Bremner WJ. Acyline-induced hypogonadism rapidly increases high-density lipoprotein cholesterol, low-density lipoprotein buoyancy and insulin levels. *W Soc Clin Investigation* Abstract # 2003
17. **Amory JK**, Watts NB, Easley KA, Sutton PR, Anawalt BD, Matsumoto AM, Bremner WJ, Tenover JL. Testosterone plus finasteride for 3 years increases bone mineral density without increasing prostate volume in older men with low serum T. *American Society for Andrology*, 2003, Abstract #10 (Platform presentation).
18. Coviello KL, Herbst KL, **Amory JK**, Anawalt BD, Yan X, Brown T, Wright B, Bremner WJ, Matsumoto AM, Jarow JP. Exogenous testosterone plus levonorgestrel profoundly suppresses intratesticular testosterone and androgenic bioactivity. *American Society for Andrology*, 2003, Abstract #96.

19. Page ST, Herbst KL, **Amory JK**, Coviello AD, Anawalt BD, Matsumoto AM, Bremner WJ Short term effects of androgen manipulation on adiponectin in normal men *Journal of Investigative Medicine*. 52 (1): S97-S98 JAN 2004
20. Coviello AD, Herbst KL, **Amory JK**, Anawalt BD, Sutton PL, Wright WW, Brown T, Yan X, Bremner WJ, Matsumoto AM, Zirkin BR, Jarow JP Low dose human chorionic gonadotropin maintains intratesticular testosterone in normal men following gonadotropin suppression. *Journal of Investigative Medicine*. 52 (1): S99-S99 JAN 2004
21. Coviello AD, Herbst KL, **Amory JK**, Anawalt BD, Jarow JP, Brown T, Wright W, Bremner WJ, Matsumoto AM Intratesticular testosterone concentrations comparable to serum levels are not sufficient to maintain normal sperm production in men. *Journal of Investigative Medicine* 51: 365 Suppl. 1 FEB 2003
22. Herbst KL, Coviello AD, **Amory JK**, Anawalt BD, Murdoch SJ, Brunzell JD, Bremner WJ. Acyline-induced hypogonadism rapidly increases HDL cholesterol, LDL buoyancy and insulin levels. *Journal of Investigative Medicine* 51: 410 Suppl. 1 FEB 2003
23. Page ST, **Amory JK**, Bowman ED, Anawalt BD, Matsumoto AM, Bremner WJ, Tenover JL. Exogenous testosterone or testosterone with finasteride improves physical performance and increases lean body mass in older men with low serum testosterone. International conference on endocrinology, 9/04.
24. **Amory JK**, Page S, Bremner WJ. Absorption of oral testosterone in oil is augmented by 5a reductase inhibition in man. *American Society of Andrology* 4/05 (oral presentation).
25. Page ST, Lin D, Nelson P, **Amory JK**, Matsumoto AM, Bremner WJ. The effect of medical castration on hormones PSA and prostate size in normal middle-aged men. *Endocrine Society* 2005.
26. Page ST, **Amory JK**, Anawalt BD, Matsumoto AM, Brockenbrough AT, Irwig MS, Bremner WJ. Is there a role for GnRH antagonists in male hormonal contraception? *Journal of Investigative Medicine* 54:S95.
27. **Amory JK**, Muller C, Page ST, Pagel E, Bhandari A, Leifke E, Bone W, Radlmier A, Bremner WJ. The effect of miglustat on spermatogenesis in normal men: A pilot study. ENDO 88th Annual meeting,
28. Page ST, Bremner WJ, Clark RB, Bush MA, Carifcofe R, Smith PM, Amory JK. Oral nanomilled testosterone (T) plus dutasteride normalizes serum T in medically castrated men: A pharmacokinetic study. ENDO 88th Annual meeting.
29. **Amory JK**, Page ST, Anawalt BD, Coviello AD, Matsumoto AM, Bremner WJ. Elevated serum INSL3 is associated with failure to completely suppress spermatogenesis in men receiving male hormonal contraception. ASA meeting 4/22/07
30. **Amory JK**, Coviello AD, Page ST, Anawalt BD, Bremner WJ. Serum 17-hydroxyprogesterone strongly correlates with intratesticular testosterone in gonadotropin-suppressed normal men receiving various dosages of human chorionic gonadotropin ENDO 89th annual meeting

Mentoring

1. Kati Matthiesson MD, 2003-2004, Effect of acyline (GnRH antagonist) on spermatogenesis, gene expression and tissue hormone levels, Monash University (PhD) candidate
2. Lindsay Bunk, MPH, 2004-2005, Acceptability of testosterone gel for male hormonal contraception, Public Health.
3. Women's Reproduction Health Research (K-12) Mentor 2005-current

Selected Presentations:

1. Amory JK. Medical Abortion. Medicine Service Conference, UCSF, 5/96
2. Amory JK. The development of the oral contraceptive for women and prospects for a pill for men. Medicine Service Conference, UCSF, 5/97
3. Amory JK. Pre-operative Evaluation. Internal Medicine Ambulatory care conference. University of Washington. 9/4/97.
4. Amory JK. Pre-operative cardiac evaluation. Chief of Medicine Rounds, VA-Puget Sound, University of Washington, 2/20/98
5. Amory JK. Pre-operative evaluation of abnormal coagulation tests. Chief of Medicine Rounds, VA Puget Sound, University of Washington, 4/28/98
6. Amory JK. Pre-operative Evaluation. General Medicine Conference, University of Washington, 9/98.
7. Amory JK. Update on hormonal male contraceptives. Reproductive Endocrinology seminar, University of Washington, Department of OB/GYN-10/23/98
8. Amory JK. An analysis of the HERS studies. Women's Health Rounds 10/19/98
9. Amory JK. Unintentional Weight Loss. Chief of Medicine Rounds, VA-Puget Sound Health Care System, University of Washington, 9/8/98
10. Amory JK. Management of patients with angina in need of non-cardiac surgery. Chief of Medicine Rounds. VA-Puget Sound Health Care System, University of Washington. 1/12/99.
11. Amory JK. Hematuria. Chief of Medicine Rounds. VA-Puget Sound Health Care System, University of Washington. 9/99
12. Amory JK. Pre-operative assessment of the geriatric patient. Department of Geriatrics grand rounds. Harborview Medical Center and University of Washington. 1/00
13. Amory JK. Pre-operative medical assessment. Noon conference. Harborview Medical Center, 8/14/00
14. Amory JK. Oral Contraceptives. Primary Care Conference, UWMC 9/17/00.
15. Amory JK. Hepatopulmonary Syndrome, UWMC, 9/13/01
16. Amory JK. Pre-operative evaluation. ACP Annual Meeting, Seattle, WA, 10/20/01
17. Amory JK. Male Contraception. Urology Grand Rounds 2/14/02
18. Amory JK. Newer contraceptives for women. Primary Care Conference 3/7/02
19. Amory JK and Hirsh I. Outpatient Management of Diabetes. Chairman's Rounds 3/20/02

20. Amory JK. Male Contraception and Infertility. Ob/Gyn Grand Rounds 5/1/02
21. Amory JK. Clostridium Difficile Colitis. Chairman's Rounds 6/11/02
22. Amory JK. Update in Contraception. Primary Care Conference 8/29/02
23. Amory JK. Peripheral Vascular Disease. Resident's Teaching Conference 9/19/02
24. Amory JK. Thyroid Function Tests: A Practicum. Primary Care Conference 9/19/02
25. Amory JK. TPMT deficiency and pancytopenia. Chairman's Rounds 10/29/02
26. *Amory JK. George Washington's Infertility: Why the father of our country was never a father. **Medicine Grand Rounds** 10/31/02
27. Amory JK. Klinefelter' Syndrome. Pediatric Endocrine Rounds. Children's Hospital, Seattle, 11/21/02.
28. Amory JK. University of Washington CPC: 23 yo male with diarrhea and Orthostasis (Autoimmune Polyglandular Syndrome). 11/20/02
29. Amory JK. Harborview Medicine CPC: 36 yo male with itching and lymphadenopathy (sarcoidosis). 11/27/02.
30. Amory JK. George Washington's Infertility. King County Medical Society. 3/12/03.
31. Amory JK. Vitamin Deficiencies in Outpatient Medicine, Chairman's Rounds, 7/17/03
32. Amory JK. Female reproductive disorders. VA endocrine conference 8/23/03
33. Amory JK. Thyroid function tests: A practicum. UW resident conference 8/27/03
34. Amory JK. Male Contraception: 2003. Association of Reproductive Professionals National Conference, La Jolla, CA (Plenary session) 9/10/03
35. Amory JK. Peripheral Vascular Disease. Resident Teaching Conference-Providence Hospital. 9/22/03 and University of Washington 10/13/03
36. Amory JK. Androgens in the Aging Male. Geriatrics Grand Rounds, Harborview Hospital, 11/7/03
37. Amory JK. Medical Jeopardy, UW resident conference, 1/9/04.
38. Amory JK. Female Reproductive Disorders. UW Primary Care Conference 4/15/04
39. Amory JK, Linden H. Breast cancer: screening, diagnosis and treatment. UW Chairman's Rounds 6/15/04.
40. Amory JK. Thyroid Function Tests, UW resident conference, 7/7/04
41. Amory JK. Vascular Disease, UW Primary Care Conference, 8/26/04
42. Amory JK. Male Contraception: Update 2004, Seattle Gynecological Society Fall Assembly, 9/10/04
43. Amory JK. "Looking up the Kilt" Adventures in Andrology Research" Helen and Phillip Fialkow Award Presentation. **Medicine Grand Rounds**, 10/28/04.
44. Amory JK. Male contraception and infertility. MEDEX grand rounds 1/3/05
45. Amory JK. Female gonadal disorders. Med 556 "Endocrinology" 1/5/05
46. Amory JK. Vitamin Deficiencies. Resident Lunch Conference, 3/2/05
47. Amory JK. Male and Female gonadal disorders. Resident teaching conf, 3/3/05

48. Amory JK. Update in Andrology. Current Concepts in Drug Therapy 3/24/05
49. Amory JK. Absorption of oral testosterone in oil is augmented by 5alpha reductase inhibition in man. American Society of Andrology Annual Meeting, Platform Presentation 4/3/05
50. Amory JK. New Developments in Male Contraception. Symposium speaker (S-39C). Endocrine Society Annual Meeting 6/3/05.
51. Amory JK. Hot Flashes in an Elderly Male. Chairman's Rounds. UW Medical Center 7/19/05.
52. Amory JK. Thyroid Disease. ACP review course 7/21/05. Seattle, WA
53. Amory JK. Peripheral Vascular Disease. Resident's noon conference, 9/1/05, Seattle, WA
54. Amory JK. Serious Adverse Medication Events. Resident's noon conference, 9/15/05, Seattle, WA
55. Amory JK. A pill for him: progress towards oral androgen therapy and oral hormonal contraceptives for men. "New Frontiers in Clinical Research: From Diagnosis to Therapy, 1st annual General Clinical Research Center Symposium, Seattle, WA 9/23/05
56. Amory JK. Postpartum Endocrinology. Endocrine Days, Semiahoo, WA 9/24/05
57. Amory JK. Thyrotoxicosis Factitia. Chairman's Rounds, UW Medical Center. 10/4/05
58. Amory JK. Update on GnRH antagonists for male contraception. 9th Summit Meeting on Hormonal Male Contraception, Geneva, Switzerland 9/11/05
59. Amory JK. Male infertility and contraception. Endocrine Teaching Conference, Harborview Medical Center, 11/22/05
60. Amory JK. Male infertility. ENDO days 1/29/06
61. Amory JK. Male infertility and contraception. Obstetrics and Gynecology Grand Rounds, University of Washington. 3/29/06
62. Amory JK. Serious adverse drug effects. UW Capstone course 5/17/06
63. Amory JK. Male Contraception. "Meet the Professor" ENDO 88th Annual meeting, Boston, MA, June 24th, 2006
64. Amory JK. Serious Back Pain. Chairman's Rounds, UWMC 7/11/06
65. Amory JK. Thyroid Disease. ACP review, Bellevue, WA 7/27/06
66. Amory JK. Peripheral vascular disease. Noon conference. Providence 8/2/06
67. Amory JK. Thyroid function tests. Noon Conference Providence 9/6/06
68. Amory JK. Peripheral vascular disease. Primary care conference 9/7/06, UWMC
69. Amory JK. Serious adverse drug effects. Noon conference. UWMC 2/14/07
70. Amory JK. Male reproductive disorders. UWMC 3/1/07,

EXTRACURRICULAR ACTIVITIES

1983 Eagle Scout
1988 & 1989 Stroke, Harvard Varsity Heavyweight Crew, Collegiate National Champions
2004 Finisher, Grand Coulee Ironman, (1st in Clydesdale Division)

Continuing Medical Education:

| | |
|--|-------------------|
| ENDO 2006-6/06 | 20 hours cat I |
| 31 st Annual Meeting of American Society of Andrology 4/06 | 16 hours cat I |
| 30 th Annual Meeting of American Society of Andrology-4/05 | 16 hours cat I |
| 29 th Annual Meeting of American Society of Andrology-4/04 | 16 hours cat I |
| 28 th Annual Meeting of American Society of Andrology-4/03 | 16 hours cat I |
| ICM II clinical tutor-2002 | 100 hours cat III |
| 27 th Annual Meeting of American Society of Andrology-4/02 | 16 hours cat I |
| ICM II clinical tutor-2001 | 75 hours cat III |
| VII International Congress of Andrology-6/01 | 20 hours cat I |
| ICM II clinical tutor-2000 | 75 hours cat III |
| "Ethical Conduct of Research with Humans"-10/3/00 | 2 hours credit |
| 25 th Annual Meeting of American Society for Andrology-4/00 | 23 hours cat I |
| ICM II clinical tutor-1999 | 75 hours cat III |
| A guide to Gulf War Veterans Health-11/98 | 6 hours cat I |
| 24 th Annual Meeting of the American Society for Andrology 4/99 | 24 hours cat I |
| ICM II clinical tutor-1998 | 75 hours cat III |
| Medical Care of Persons with Spinal Cord Injury 11/98 | 10 hours cat I |

Bicycling

Floyd's Performance: Stage 1

Flat course makes aerodynamic drag an important factor in outcome for Floyd Landis and others

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Stage 1: Saturday, July 2

Route: Fromentine to Noirmoutier-en-l'Île

Type: Time Trial

Distance: 19 Km

Elevation Gain: 0 feet

Total Feet Climbed: 66 feet (20 meters)

At a Glance:

Stage Placing: 6th

GC Placing: 6th

Time: 00:21:53 (00:01:09 down on Dave Zabriskie)

Estimated Average Power: 443 Watts

Estimated Total Work: 582 Kjoules

Stress to Strain Index: 1.13

Today's stage from Fromentine to Noirmoutier-en-l'Île was a nearly perfectly flat 19 kilometer (11.78 miles) time trial with a strong head and cross wind over much of the course. Because we will not be monitoring Floyd's power output during the time trials, I've estimated, based on Floyd's aerodynamic drag, what we believe his average power output was over today's stage. In addition based on the height and weight of his competitors and a mathematical model developed from their previous time trial results, I've come up with an estimate for the power outputs of the rest of the top 10 (Table 1).

On a flat course the primary variable resisting a cyclist's forward motion is aerodynamic drag or wind resistance. Thus, on today's stage a rider's power output is basically equal to their speed multiplied by aerodynamic resistance. Going fast on today's stage not only required a huge amount of power, but also great aerodynamics. In fact, among professional cyclists, on a perfectly flat course, aerodynamics is probably a stronger determinant of speed than their power output.

For example, at 30 mph, an increase in aerodynamic load of only 100 grams or just under a quarter of a pound can increase the power requirement by 15 Watts. That's equivalent to an extra 5 pounds on a 6 percent grade hill and would result in a 45 second time loss over an hour. In today's time trial, if the aerodynamic drag on Dave Zabriskie increased by 100 grams, he would have lost the time trial by about 20 seconds. That's 0.2 seconds per gram. It's interesting to note, that Dave's aerodynamic drag at his speed was about 185 grams less than Lance Armstrong's. Though Lance probably produced the highest average power output of the day, he lost to Dave by only 2 seconds or about 10 grams of aerodynamic drag. If we assume all the riders went 30 mph, then the actual difference in aerodynamic drag between Dave and Lance works out to be only 83 grams, the difference between Dave and Floyd is 44 grams, and Dave and Vinokourov is 48 grams, with Dave more aerodynamic than all of the riders in the top 10. That aerodynamic drag combined with an incredibly impressive power output won the day for Dave, who is likely to only become a stronger rider in the years to come. For a closer look at the effect of body position and speed on aerodynamic drag and power output see Table 2.

For Floyd, the day went quite well and he is extremely happy with his ride. More importantly, he's ecstatic about Dave's performance. Floyd and Dave share an apartment in Girona, Spain, where they live while in Europe. After Dave returned from the Giro D'Italia, Floyd encouraged him to focus strongly on this opening time trial stage, clearly believing that Dave could easily win. Although, the short time trial is not one of Floyd's specialties, among the potential contenders for the overall, he is currently third behind Lance Armstrong and Alexander Vinokourov. Despite the lost time, this race is still wide open with plenty of hard days ahead.

| | Rider Name | Time min:sec | Speed | | Drag N/V ² | Power Watts | Power W/kg | Work Kj |
|----|----------------------|-----------------|-------|------|--------------------------|----------------|---------------|------------|
| | | | kph | mph | | | | |
| 1 | David Zabriskie | 20:51 | 54.68 | 33.9 | 0.121 | 450 | 6.52 | 626 |
| 2 | Lance Armstrong | 20:53 | 54.59 | 33.8 | 0.123 | 476 | 6.61 | 646 |
| 3 | Alexandre Vinokourov | 21:44 | 52.45 | 32.5 | 0.133 | 455 | 6.32 | 593 |
| 4 | George Hincapie | 21:48 | 52.29 | 32.4 | 0.138 | 473 | 5.93 | 663 |
| 5 | Laszlo Bodrogi | 21:50 | 52.21 | 32.4 | 0.124 | 422 | 6.03 | 593 |
| 6 | Floyd Landis | 21:53 | 52.09 | 32.3 | 0.122 | 414 | 6.08 | 582 |
| 7 | Fabian Cancellara | 21:54 | 52.05 | 32.3 | 0.135 | 455 | 5.83 | 641 |
| 8 | Jens Voight | 21:55 | 52.02 | 32.2 | 0.125 | 421 | 6.01 | 593 |
| 9 | Vladimir Karpets | 21:58 | 51.90 | 32.2 | 0.138 | 461 | 5.84 | 651 |
| 10 | Igor Gonzalez | 21:59 | 51.86 | 32.2 | 0.133 | 445 | 5.80 | 650 |

Table 1. Estimated power outputs, power to weights, and total work done for today's top ten calculated from the finishing time and an estimate of aerodynamic drag (Drag in Newtons per velocity squared adjusted for wind speed) based on each rider's height, weight, and a mathematical model developed from previous time trial finishes.

©Allen Lim

GDC00792

Bicycling

Floyd's Performance: Stage 2

Learn how Landis' stress to strain index is keeping him right on target

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Challans to Les Essarts
Type: Flat Road Race
Distance: 181.5 km
Elevation Gain: 115 meters or 377 feet
Total Feet Climbed: 66 feet or 20 meters

At a Glance:

Stage Placing: 113th
GC Placing: 6th
Time: 03:51:05 (00:00:05 down on Tom Boonen)
Average Power: 206 Watts
Total Work: 2885 Kjoules
Stress to Strain Index: 0.97

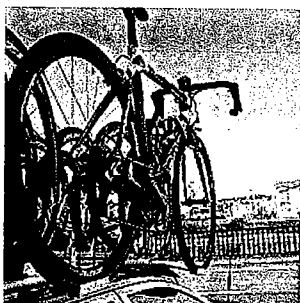
Stimulus vs. Response:

With a gain of only 115 meters over 181.5 kilometers or an average percent grade of 0.06%, today's stage was definitely flat, with only minor rollers over the last 30 kilometers. And so it was a day for the sprinters allowing the major GC riders like Floyd to sit in and conserve as much energy as possible.

This year, there's a new rule at the Tour for these flat stages. The finishing time is taken 3 km from the finish so that the riders who don't want to risk fighting it out in a field sprint can sit back and let the sprinters go. For Floyd, this meant that he could relax and hang in the back through the small and twisty roads approaching the finish. With more and more traffic circles dotting the landscape of France it's getting harder and harder for Tour organizers to find a straight and safe line into the small towns where many of the stages finish. The new rule helps to alleviate some of the danger and allows riders like Floyd to focus on two things during these opening stages - conserve and stay out of trouble.

Based on today's power data (**Table 1**), Floyd did a great job of conserving. With an average power output of only 206 watts and a total workload of just 2885 Kjoules, today's race was significantly easier for Floyd than any 4 hour training ride that he'd do alone.

On a rating of perceived exertion scale of 1 to 10 (**Table 2**) Floyd gave the day a 4. We use this rating to determine the stress to strain index listed above which is a simple way to gauge how he's holding up during training and racing. The index is derived from previous testing we have done that describes the relationship between his perceived exertion and his power output when he's fresh and in form. Thus, we use the rating to get a prediction of what he thought or felt his average power was for the day. For Floyd, a rating of 4 gives us a prediction of 200 Watts, which is only 6 watts lower than his actual power of 206 Watts. Since the average power multiplied by time gives us the total work done or energy used, we can also take Floyd's estimated power of 200 Watts and calculate how much work or energy he felt he used - a prediction of 2772 Kjoules or 113 Kjoules less than what he actually did. The index is just the ratio between what he felt his power output or work was and what his actual power output or work was.



©Allen Lim

This distinction between what Floyd actually did versus what he felt he did is an extremely important one. It's what many physiologists term a "Stimulus" vs. "Response" or "Stress" vs. "Strain" relationship. In Floyd's case there's what really happened as measured by the PowerTap ("Cause") and then there's what he thinks happened based on his own perception and feel ("Effect"). To really understand a training or race situation you need to know both.

On a rating of perceived exertion scale of 1 to 10 Floyd gave the day a 4. We use this rating to determine the stress to strain index listed above which is a simple way to gauge how he's holding up during training and racing. The index is derived from previous testing we have done that describes the relationship between his perceived exertion and his power output when he's fresh and in form. Thus, we use the rating to get a prediction of what he thought or felt his average power was for the day. For Floyd, a rating of 4 gives us a prediction of 200 Watts, which is only 6 watts lower than his actual power of 206 Watts. Since the average power multiplied by time gives us the total work done or energy used, we can also take Floyd's estimated power of 200 Watts and calculate how much work or energy he felt he used - a prediction of 2772 Kjoules or 113 Kjoules less than what he actually did. The index is just the ratio between what he felt his power output or work was and what his actual power output or work was.

This distinction between what Floyd actually did versus what he felt he did is an extremely important one. It's what many physiologists term a "Stimulus" vs. "Response" or "Stress" vs. "Strain" relationship. In Floyd's case there's what really happened as measured by the PowerTap ("Cause") and then there's what he thinks happened based on his own perception and feel ("Effect"). To really understand a training or race situation you need to know both.

Table 2. A modified Borg perceived exertion scale used to get a quantifiable number attached to an athlete's subjective rating of how they felt during a given training ride or race. Because the scale is simply an intensity scale and since the total work done or energy expended is equal to intensity multiplied by time, an easy way to get a relative measure of how hard you worked on a given day is to multiply your exercise time in minutes by your perception of effort on a 1 to 10 scale.

As an example, on one day you

GDC00793

Table 1. Floyd's power summary for Stage 2. Based on today's numbers, Floyd had a relatively easy day on the bicycle. His average power output and work are well below his training averages for the duration. His stress to strain index is just below 1.0. Peak power outputs are all lower than his personal bests and training averages. Finally, he distributed the majority of his time below his lactate threshold (an intensity he considers "hard"). In fact, over the course of today's ride, he spent 16.1% or 37 minutes not even pedalling.

| | Stage 2 7/23/05 | Tour de France Average (excluding TT's) |
|-----------------------------|-----------------------|--|
| Duration | 3:51:36 (hrs:min:sec) | 3:52 ± 00.00 (hrs:min) |
| Distance | 181.5 Km or 113 miles | 113 ± 0 miles |
| Average Speed | 29.2 mph | 29.2 ± 0 mph |
| Perception of Effort (1-10) | 4 | 4 ± 0 |
| Average Power | 206 Watts | 206 ± 0 Watts |
| Total Work from Power | 2885 Kjoules | 2885 ± 0 Kjoules |
| Total Work from RPE | 2772 Kjoules | 2772 ± 0 Kjoules |
| Stress to Strain Index | 0.97 | 0.97 ± 0 |
| Peak Power for 5 sec | 830 watts | 830 ± 0 watts |
| Peak Power for 30 sec | 465 watts | 465 ± 0 watts |
| Peak Power for 1 min | 403 watts | 403 ± 0 watts |
| Peak Power for 5 min | 308 watts | 308 ± 0 watts |
| Peak Power for 30 min | 283 watts | 283 ± 0 watts |
| Peak Power for 1 hour | 231 watts | 231 ± 0 watts |
| Peak Power for 2 hours | 214 watts | 214 ± 0 watts |
| Time at Zero Watts | 16.1% or 37 min | 16.1 ± 0% or 37 ± 0 min |
| Time in Recovery Zone | 51.7% or 73 min | 51.7 ± 0% or 73 ± 0 min |
| Time in Endurance Zone | 31.0% or 72 min | 31.0 ± 0% or 72 ± 0 min |
| Time in L.T. Zone | 7.3% or 17 min | 7.3 ± 0% or 17 ± 0 min |
| Time in Race Pace Zone | 5.1% or 12 min | 5.1 ± 0% or 12 ± 0 min |
| Time in Max Zone | 3.4% or 8 min | 3.4 ± 0% or 8 ± 0 min |
| Time in Supra-max Zone | 5.4% or 12 min | 5.4 ± 0% or 12 ± 0 min |

©Allen Lim

know he's doing what he's suppose to be doing at this stage of the game. As he fatigues and we start to approach the really difficult stages in the mountains, I suspect that the ratio will increase well above 1 to values close to 2.

While this may seem a little complicated it is just another way to quantify the simple question, "How'd you feel today." After all the sophisticated analysis and technology, it's the first thing every coach, including myself, asks. Over the course of the Tour, I'll talk about more of the variables listed in the table below and share more of the subtle tricks and basic principles we use to help Floyd perform optimally. For now, he's right on target. He's conserving and staying out of trouble. More importantly reality and his perception of reality are right in line.

Dr. Allen Lim is an exercise physiologist and coach with Thrive Health Fitness Medicine (www.thrivehfm.com). He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising Floyd Landis (www.floydlandis.com) bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

1-10 RPE Scale

| | |
|----|------------------------------------|
| 0 | Rest |
| 1 | Really Easy |
| 2 | Easy |
| 3 | Moderate |
| 4 | Sort of Hard |
| 5 | Hard |
| 6 | |
| 7 | Really Hard |
| 8 | |
| 9 | Really, Really, Hard |
| 10 | Maximal: Just like my hardest race |

©Allen Lim

what an athlete actually does should be equal to 1. If that athlete thinks the ride is easier than it actually is the ratio would dip below 1. On the other hand, if an athlete thinks the ride is harder than it actually is, the ratio would be greater than 1. Thus, in addition to tracking the average intensity of each day in Watts and the total energy or work done in Kjoules, we also measure Floyd's Stress to Strain Index. At an index just below 0.97, he's just below what an athlete perceives and

may feel great at an average power of 150 Watts, but on the very next day you may feel terrible at the same power output. Since the power output is the same, there has to be something explaining the difference in perception. That something could include factors like fatigue, dehydration, or differences in the environment. Whatever the cause, being able to track the offset between the actual power and the rider's perception of power can give us important insight into the condition or state of an athlete that we wouldn't normally have with either variable alone. If everything is normal, then the ratio between

Bicycling

Floyd's Performance: Stage 3

Saving Energy: Different Spokes for Different Folks

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: La Chataigneraie to Tours
Type: Flat Road Race
Distance: 212.5 km
Elevation Change: - 33 meters or - 108 feet
Total Feet Climbed: 388 meters or 1,273 feet

At a Glance:

Stage Placing: 131th
GC Placing: 6th
Time: 04:36:09 (Same time as winner Tom Boonen)
Average Power: 226 Watts
Total Work: 3743 Kjoules
Stress to Strain Index: 0.88

It was another day for Tom Boonen who's sprinting form is definitely on. With today's win, his confidence is sure to rise and this won't be the last time we see him cross the line first.

The goal for Floyd today was to try and hold an even lower power output than yesterday. At least, that's what we joked about in the bus this morning. Though it's fun to make it a game, conserving energy in these early stages is serious business and will be a critical factor for tomorrow's team time trial and the mountains next week.

There are a couple of things Floyd tries to do to save energy on these early stages. First and foremost, he is never in the wind. Never. If he needs to move up, he either carves his way through the pack or has his teammates pull him up the side. By drafting in the pack, Floyd can save between 40 to 50% of the energy he would be using if he were in the wind. As an example, in yesterday's stage if Floyd did the race by himself or at the front, his average power would have been 410 Watts vs. the 206 Watts he actually did. For all practical purposes, sitting in yesterday saved Floyd 50%.

Another tactic, though somewhat dangerous, is drifting back on the climbs. That is, Floyd will move to the front of the pack before a slight roller or climb and then over the course of that climb, he'll ease up and let himself drift towards the back. If he times it right, then by the time the pack crests the top of the hill, he'll be towards the rear. While it's not always wise to get caught in the back, soft-pedaling the hills not only saves a lot of energy, it's also easier on the body to ride a steady pace rather than a sporadic one.

On the flat or downhill portions, another factor that Floyd tries to stay conscious of is not pedaling. It may sound silly, but over the course of a race a skilled cyclist can coast 10 to 20% of the time. In a 5-hour road race, not pedaling for 20% of the time is equal to not pedaling for 1 hour of that race. Those periods of recovery can save a tremendous amount of energy helping to cut a significant number of miles out of the legs.

One interesting tactic we've adopted specifically for Floyd and his teammates this year is selecting different wheels for different riders based on their specific role for the day. There are a number of different factors resisting a cyclists' movement. Those factors include gravitational resistance (i.e., hills), aerodynamic resistance (i.e., wind), frictional resistance (i.e., bearings & chain), and inertial resistance (i.e., accelerations). On a flat course like today, the two most important factors to consider are aerodynamic resistance and inertial resistance. Thus, our goal is to choose wheels that cheat the wind and that are easy to accelerate. While being more aerodynamic makes intuitive sense, what many people don't realize is that in the middle of a moving pack, where aerodynamics is not as large of a factor, a rider will surge thousands of times, literally between every other pedal stroke, in order to maintain position - mere inches away from the wheel in front. The question at hand is how do we optimize the energy savings for the riders who's job it is to protect Floyd, for Robbie Hunter—the team's sprinter, and of course for Floyd.

To that end, the team is lucky enough to be sponsored by Zipp— a U.S. wheel manufacturer that has not only provided one set of wheels to the team but five different types of wheels, each with different rim depths and weights that allows us to minimize the power cost for each rider.

For Robbie, his goal is to go as fast as possible in the final sprint. Because aerodynamic resistance increases exponentially with speed and since Robbie's job is to go the fastest, he rides the 808 wheel set, which with the deepest depth rim is the most aerodynamic. For the riders protecting Floyd, they need a wheel that is light to help them with the accelerations and aerodynamic since they're in the wind more than Floyd. To that end, they ride the 404 wheel set that is lighter than the 808 but not as deep and thus slightly less aerodynamic.

Finally, because Floyd is so well protected, the aerodynamics of his wheels are not as important as the other riders. What matters more is saving energy by minimizing the cost of the accelerations he makes in the pack. Accordingly, Floyd rides the 202 wheel set that are some of the lightest wheels on the market. Though they are not as aerodynamic as the 808's or 404's, they are still significantly more aerodynamic than a standard 32 spoke wheel (Table 1 & Table 2). In the end, it's about balancing aerodynamic resistance with inertial resistance. Since no single rider has the same role on the team, they each use a wheel set that suits their specific needs.

Table 1. The power required to go 30 mph using a standard 32 spoke wheel versus various Phonak Team Zipp wheels at different wind angles.

Table 2. The power savings at a given wind angle between the Phonak Team Zipp wheels and a standard 32 spoke wheel.

Our hope is that these subtle tactics will help give Floyd and his entire team an edge in tomorrow's team time trial as well as the rest of the race. It's a long haul to Paris, and every bullet saved now could

GDC00795

| | | Power (Watts) at 30 mph | | |
|-------|-----------|-------------------------|-----|-----|
| | | Wind Angle | | |
| Wheel | 32 Spoke | 0° | 15° | 30° |
| | 202 Zipp | 437 | 445 | 480 |
| | 404 Zipp | 427 | 425 | 435 |
| | 808 Zipp | 422 | 413 | 427 |
| | Disc Zipp | 421 | 409 | 425 |

©Allen Lim

| | | Power (Watts) Savings Compared to a Standard 32 Hole Spoke Rim at a Given Wind Angle | | |
|-------|-----------|---|-----|-----|
| | | Wind Angle | | |
| Wheel | 202 Zipp | 0° | 15° | 30° |
| | 404 Zipp | 10 | 20 | 45 |
| | 808 Zipp | 15 | 32 | 53 |
| | 808 Zipp | 16 | 36 | 55 |
| | Disc Zipp | 17 | 38 | 78 |

©Allen Lim

be a bullet that saves their lives later.

Based on Floyd's power data (Table 3), he had what could be considered another easy day on the bike today. He didn't achieve the goal of riding a lower average power output but it would've been hard with the extra rollers in today's stage. Those rollers and the fact that he didn't spend as much time not pedaling raised his average power. Also, the last hour of today's stage was especially fast and he had to bring it up a notch over the last hour, which not only increased his average power but also caused him to accumulate a bit more time at lactate threshold and above. On the other hand, his stress to strain index was lower today than it was yesterday. A good sign he feels good and is ready to go.

Table 3. Floyd's power data from stage 3 as well as the Tour average and the current minimum and maximum for the last two days.

| | | Stage 3 | Tour Average | Min | Max |
|-------------------|------------------------------|-------------|------------------------|-------------|-------------|
| Basic Stats | Duration (hrs:min) | 4:16 | 4:14 ± 00:52 | 3:51 | 4:39 |
| | Distance (miles) | 132 | 122 ± 14 | 113 | 122 |
| | Average Speed (mph) | 28.6 | 28.9 ± 0.4 | 28.6 | 29.2 |
| | Perception of Effort (1-10) | 4 | 4.0 ± 0 | 4 | 4 |
| | Average Power (Watts) | 226 | 216 ± 14 | 206 | 216 |
| | Work from Power (Kjoules) | 3743 | 3599 ± 628 | 3895 | 3743 |
| Peak Power | Work from RPE (Kjoules) | 3312 | 3042 ± 382 | 2722 | 3312 |
| | Stress to Strain Index | 0.88 | 0.75 ± 0.06 | 0.88 | 0.97 |
| | 5 sec (Watts) | 915 | 882 ± 75 | 830 | 935 |
| | 30 sec (Watts) | 525 | 497 ± 45 | 465 | 528 |
| | 1 min (Watts) | 416 | 410 ± 38 | 403 | 456 |
| | 5 min (Watts) | 383 | 346 ± 53 | 308 | 383 |
| Zone Distribution | 10 min (Watts) | 315 | 282 ± 47 | 284 | 345 |
| | 1 hour (Watts) | 278 | 255 ± 33 | 231 | 278 |
| | 2 hours (Watts) | 249 | 238 ± 16 | 214 | 249 |
| | Zero Watts (% or min) | 10.5% or 29 | 17.1 ± 6.2% or 24 ± 21 | 10.5% or 29 | 16.1% or 37 |
| | Recovery (% or min) | 29.0% or 30 | 26.3 ± 7% or 33 ± 26 | 29% or 37 | 31.7% or 33 |
| | Endurance (% or min) | 55.5% or 99 | 50.5 ± 2.4% or 95 ± 9 | 51% or 22 | 56% or 99 |
| Zone Distribution | Lactate Threshold (% or min) | 8.2% or 23 | 9.1 ± 4.3% or 28 ± 14 | 7.3% or 17 | 8.2% or 23 |
| | Race Pace (% or min) | 6.4% or 18 | 7.3 ± 3.9% or 23 ± 12 | 5.1% or 12 | 6.4% or 18 |
| | Stress (% or min) | 4.5% or 12 | 3.6 ± 2.4% or 13 ± 6 | 3.1% or 7.9 | 4.5% or 12 |
| | Supra-Max (% or min) | 5.5% or 16 | 5.6 ± 1.3% or 17 ± 4 | 5.4 or 12.5 | 5.8% or 16 |

©Allen Lim

Bicycling

Floyd's Performance: Stage 4

Powerfeed: Landis' team lays it on the line

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Tours to Blois

Type: Team Time Trial

Distance: 67.5 km

Elevation Change: 29 meter gain or 93 feet gain (Average grade = 0.04%)

Total Feet Climbed: 124 meters or 407 feet

Weather: 75° F with overcast skies & cooling off. Perfect riding conditions.

At a Glance:

Stage Placing: 5th

GC Placing: 20th

Time: 01:12:10 (1:31 down on Discovery)

Estimated Average Power for Floyd Pulling: 557 Watts for 1 minute (14.4 min total)

Estimated Average Power for Floyd Drafting: 334 Watts for 4 minutes (57.7 min total)

Overall Estimated Average Power: 379 Watts

Total Work: 1641 Kjoules

Stress to Strain Index: 1.32

It wasn't a bad ride for the Phonak boys, but as team time trial specialists, they had high expectations. While they might be somewhat disappointed, it was still a solid performance—one that required a combination of tremendous power and skill.

Team Time Trial Strategy:

This afternoon, before the race, the boys got together at lunch to do some last minute review of their team trial strategy. Long story short, the tactic was to lay it on the line and go fast. There are a couple of key rules that the team uses in that pursuit. Despite the aggression and brute power required for the team time trial, they all revolve around cooperation and letting go of ego. The basic rules are as follows:

1. Maintain a Constant Speed: A key to keeping things moving and obtaining the fastest speed on a course like today is to try and maintain the most even speed or pace possible. That is, the rider taking a pull will do his best not to try and accelerate or decelerate when he's pulling. Sudden surges within the line can really hurt the riders in the back. At the same time, any drop in speed can really hurt performance. So how do different riders maintain the same speed as the rider before them if there are different ability levels on the team? That brings us to rule 2.

2. Pull Only as Long as You Can Hold the Speed: Since the goal is to maintain an even pace, the weaker riders may only pull for short periods of time (5 to 30 seconds) while the stronger riders will pull for longer periods of time (30 seconds to 1 minute). On average a rider will only pull at the front for about 30 seconds before falling to the back to recover. It takes really knowing yourself and letting go of your pride to ride within yourself and not pull for too long. In the end, if a rider isn't honest with their efforts and blows, that's one less person in the rotation to help provide draft for the others to recover. It's a delicate balance as every rider needs to tow the line between giving an all out effort at the front while still having enough to get back on and recover. If a rider is feeling tired, they will simply move through the rotation and pull off when they reach the front. It's still faster than if that rider wasn't there. It also allows that rider to recover and contribute later in the race. Of course, the ultimate question is, who determines the speed? That brings us to rule 3.

3. Stagger Strong Riders Between Weaker Riders: On the Phonak squad riders like Santiago Botero, Robbie Hunter, Oscar Pereiro, and Floyd are the real strong men in the team time trial. Their experience and strength are used to help keep the pace high and on course. Thus, these riders are staggered between the weaker ones so that at any given point in the rotation there is a minimal amount of time between any modulations in pace. If this wasn't done and there are clusters of strong or weak riders in the line, then there would be periods of time where the pace would be too high and other periods where the pace was too low. The best team time trial riders, not only have the strength to lift the speed should it drop, but also have the skill to do it over a longer period of time, thus helping to keep accelerations in the line at a minimum. Of course, none of this would work without the last and most important rule.

4. Communicate: This is a no brainer, but when the pace is high and riders are on the edge it's hard to remember to let others know how you're feeling or to tell a rider in front if they're going too fast or too slow. From a practical perspective, there's also the need to make sure those in back know what's coming up front. What might be a common courtesy of pointing out a pothole or bump in the road on a weekend ride could be a matter of keeping the train on the tracks or derailing the entire caboose. Although the riders all know each other well and have an intuitive sense of how everyone is doing, that's the direct result of continued and constant communication.

Predicting the Days' Results:

Based on today's course profile, the team's strategy, and their finishing time, I built a mathematical model predicting how much power the average rider had to produce at the front of the line compared to what they were doing when drafting.

At the front of the Phonak line today, the average predicted power to maintain a speed of 56.1 km/hr was 557 watts. For most of the riders that's 130% of the power associated with their maximal ability to consume oxygen or VO2 max. This means, that there's no way that any of the riders could have pulled for more than 4 minutes, which is the average length of time an athlete can ride at their VO2 max. More importantly, this also means that when taking a pull at the front, they are relying heavily on energy from both aerobic and anaerobic sources. Thus, the team trial is one that requires a unique combination of both aerobic and anaerobic capacity.

While sitting in and drafting, I estimated an average power output of 334 Watts. This is about 80% of the athlete's VO2 max, which is just below their lactate threshold (LT) or an intensity they can hold for 1 to 2 hours (More about lactate threshold and VO2 max in future articles). With 8 guys pulling for an average of only 30 seconds, however, there are only 4 minutes just below LT to recover from what is essentially an all out effort.

Thus, even though the weighted average power that I've predicted is only 379 Watts for Floyd (an intensity that is lower than what he'll hold alone in an individual time trial), the team trial is an extraordinarily difficult event because of the hard surges required at the front. It's like an hour of all out interval work with barely enough time to recover. This explains why, his stress to strain index for today is significantly elevated for today's short ride at a value of 1.33.

I also couldn't help but figure what the boys needed to do today to match Discovery's impressive ride. Assuming that the overall aerodynamic drag characteristics and weight of both teams is equal, then each rider on Phonak needed to produce 5.8% more power or 32 more watts at the front and rear to match the winning time. Thus,

each of the Discovery riders had to be redlining at an average power output just below 400 watts for the one-hour duration. An effort like that is at the edge of what's humanely possible. It's an effort that I'm know Team Phonak will have their eyes on for next year.

*Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.*

Bicycling

Floyd's Performance: Stage 5

Determining Landis' Power

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Chambord to Montargis
Type: Flat Road Race
Distance: 183 km
Elevation Change: 20 meter gain or 66 feet gain (Average grade = 0.01%)
Total Feet Climbed: 307 meters or 1007 feet
Weather: 72°F. Cool and overcast with intermittent sprinkles.

Climbs:

1. Cote de Bellevue (Category 4: 1 km at 4.2%)

At a Glance:

Stage Placing: 105th
GC Placing: 20th
Time: 03:46:00 (Same time as winner Robbie McEwan)
Average Power: 220 Watts
Total Work: 2983 Kjoules
Stress to Strain Index: 1.14

Predicted Average Power at Front: 476 Watts
% Savings due to Drafting: 54%

Recap:

Before I get into today's stage, I thought I'd recap a little bit for stage 3 and 4. On stage 3 our goal was for Floyd to try and conserve even more than he did the day before. But by the end of the day he actually had a higher average power. Besides the more undulating terrain, another major factor to that higher average was a strong tailwind that pushed the peloton for most of the day.

Although we normally associate a tailwind with an easier ride, that's really only true for the person in the wind. For someone who is drafting, two things happen. First, the overall speed for any section of terrain is higher so the power required even when drafting is slightly higher. Second, the relative advantage of the draft is decreased. For these reasons, it is actually a little harder to sit in the pack. Primarily, you simply can't soft pedal as much. This was definitely reflected in the 16% time at zero watts on stage 2 vs. the 10.5% time at zero watts on the tailwind aided (or hampered) stage 3.

As for yesterday's team time trial—the good news is Floyd felt great during the race and I think he'll have excellent form in the mountains. The bad news is that the team broke almost every rule in team time trialing—and that's really the nature of the beast. It is such a delicate balance, that one mistake and the day is done. Yesterday, some of the boys were just a little too ambitious and bit off a little more than they could chew. Once that happens, there's no recovering. I think the big lesson learned, and reinforced, is that beyond trying to hold a constant speed, each rider needs to pull off the front before they're blown. For pros, that's sometimes hard to do. And that's exactly why time trialing—especially the team time trial—is an egoless event where pacing and patience are all important.

Stage 5:

From Floyd's perspective, there's not too much to report from today's stage. It was just another day of sitting and hiding in the pack and based on the power analysis (**Table 1**), things are fairly similar to the previous flat stages. In many ways, however, these flat stages are a whole new experience for him.

During his days with U.S. Postal, he spent most of these early days taking 1 to 5 minute pulls in the wind at an average of 400 Watts per pull. Those pulls are 50% more than what he's doing now in the pack. But what does riding at 400 Watts vs. 200 Watts actually mean for Floyd's body? To answer that question, we periodically test Floyd either in the lab or in the field to assess the specific physiological factors determining Floyd's ability to produce power. These same tests are also available to the general public through **Thrive Health**

Fitness Medicine

Determinants of Power:

There are 4 key factors determining a cyclist's ability to produce power. Those factors are:

1. **VO2 max:** An athlete's maximal capacity to consume oxygen.
2. **Lactate Threshold or LT:** The percentage of that maximal capacity that an athlete can hold for an extended period of time (1 to 2 hours).
3. **Efficiency:** the percent of each calorie burned that actually gets transferred to the bike
4. **Anaerobic Capacity or Power:** The maximal amount of power or energy a person can produce in a 5 second to 30 second period of time.

An athlete's VO2 max is the upper limit for how much oxygen they can consume. Since oxygen consumed by your body is directly related to how much energy you're using, VO2 max is essentially the upper limit for how much energy or power your body can generate. VO2 max for a cyclist is what maximal horsepower is for a racecar. In practical terms, an athlete's VO2 max is basically the highest power he or she can hold for about a 4-minute period of time. For Floyd, his power at VO2 max hovers between 450 and 475 watts depending on how fit or tired he is. So, if Floyd were to have taken a turn at the front today, he would've been at 476 Watts or 100% of his VO2 max. While a pull at that power output for less than 4 minutes is well within his capacity, any longer and there might be a serious detonation. That's why when you watch the guys at the front they're rarely pulling for longer than 4 minutes at a time. That's because for most of them they're close to red line when in the wind. Though everyone wants that racecar with the highest maximal horsepower, like max horsepower, VO2 max, while an important factor in an athlete's success, is by no means a make or break factor. It may be a big distinguisher between the average Tour rider and the average guy on the street. Amongst top pros, however, VO2 max is actually a poor predictor of performance.

Because VO2 max and LT are critical physiological attributes, we use them as a reference points for analyzing all of Floyd's power data (**Figure 1**). In the daily power table under "Zone Distribution" you'll notice that we break up the amount of time he spends in different intensity ranges. The first range or "zone" is simply time not pedaling. The second is what we call a "recovery zone," which is the range between zero watts and half the distance to the beginning of Floyd's LT. The "endurance zone" is just the range between the top of recovery to the beginning of LT. The "LT zone" is the power range associated with Floyd's LT. The "race pace zone" is the range between LT and VO2 max. The "VO2 max" zone is the power that elicits Floyd's top end. And finally, "Supra-Max" is anything above his VO2 max. For now, our goal is to accumulate more time in the lower zones. By the time we get into the mountains there will be a significant shift the other way.



©Allen Lim

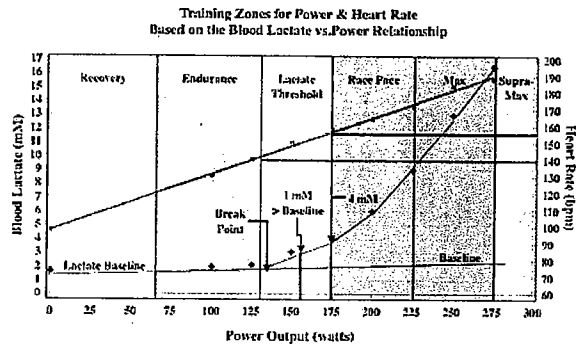
Although not as large of a determinant of performance as VO2 max and LT amongst professional cyclists, "efficiency" is still an important factor. If you've ever touched the hood of a car after driving or gotten way too hot while exercising then you've just experienced what efficiency or inefficiency is. The reason why we and your car engine gets hot is because most of the energy human and mechanical engines burn is wasted and lost as heat rather than converted to useful mechanical energy. The average cyclist is only 22% efficient on a bike. That means for every 100 Calories they burn while exercising only 22 of those Calories actually goes to the pedals to help move the bike. Some studies have shown that cyclists in the Tour de France can be as much as 27% efficient, while others studies have shown that untrained individuals can be as low as 18% efficient. This number ultimately translates to how much food they need to keep them going (more specifically on food intake during the Tour soon). The more efficient, the less food a rider needs. During the long stages of the Tour where running out of energy and bonking is a real threat, it's the more efficient rider who will have more energy reserves for the end. The last factor determining a cyclist's ability to produce power is their "Anaerobic Power or Capacity." This is how much power they can produce for very short periods of time like a sprint. You might notice that for Floyd, the highest power he's reached so far in the Tour for a 5 second period is 935 watts. In the realm of anaerobic power, that value sucks. As a reference, Robbie McEwen was probably in the 1400 to 1700 watt range in the sprint he won today. Needless to say, Floyd wasn't up there. And that's what is so interesting about the variation in physiology we have at the Tour. For guys like Floyd who possess a high LT, their anaerobic capacity tends to be terrible. Likewise, for the sprinters who have an amazing anaerobic capacity their LT values tend to barely get them over the hills. The one exception is probably Lance, who can climb with the best and sprint if pushed. In coming stages, continue to expect low 5-second peak power values from Floyd as well as higher and higher 30-minute and 1-hour power values from him when he reaches the mountains.

Table 1. Power data for Stage 5. All Tour average statistics as well as min and max values exclude time trial data. Looks like another flat stage.

| | Stage 5 | Tour Average | Min | Max |
|-------------------|------------------------------|--------------|--------------------|-------------|
| Basic Stats | Duration (hr:min) | 3:46 | 4:04 (S5) | 4:30 (S3) |
| | Distance (miles) | 113 | 119 (S1) | 132 (S3) |
| | Average Speed (mph) | 30.1 | 29.3 (S5) | 30.1 (S5) |
| | Perception of Effort (1-10) | 4.5 | 4.3 (S6) | 4.5 (S5) |
| | Average Cadence (RPM) | 86 | 88 (S2) | 90 (S2) |
| | Average Power (Watts) | 220 | 217 (S1) | 220 (S3) |
| | Work from Power (KJoules) | 2983 | 3194 (S4) | 3243 (S3) |
| | Work from RPE (KJoules) | 3390 | 3042 (S2) | 3390 (S5) |
| Peak Power | Stress to Strain Index | 1.54 | 1.00 (S1) | 1.34 (S5) |
| | 5 sec (Watts) | 795 | 553 (S4) | 935 (S3) |
| | 30 sec (Watts) | 534 | 509 (S3) | 554 (S2) |
| | 1 min (Watts) | 473 | 444 (S2) | 473 (S5) |
| | 5 min (Watts) | 331 | 341 (S1) | 383 (S3) |
| | 30 min (Watts) | 258 | 274 (S3) | 315 (S3) |
| | 1 hour (Watts) | 248 | 252 (S2) | 278 (S3) |
| | 2 hours (Watts) | 220 | 235 (S2) | 249 (S3) |
| Zone Distribution | Zero Watts (% or min) | 13.2% or 30 | 13 (S3) or 32 (S1) | 29 min (S3) |
| | Recovery (% or min) | 28.6% or 65 | 30 (S2) or 72 (S1) | 30 min (S3) |
| | Endurance (% or min) | 55.3% or 76 | 55 (S2) or 82 (S1) | 99 min (S3) |
| | Lactate Threshold (% or min) | 8.2% or 19 | 8 (S1) or 19 (S1) | 17 min (S2) |
| | Race Pace (% or min) | 6.5% or 15 | 6 (S1) or 15 (S1) | 25 (S3) |
| | Max (% or min) | 5.0% or 9 | 4 (S2) or 10 (S2) | 8 min (S2) |
| | Supra-Max (% or min) | 0.1% or 14 | 0 (S2) or 14 (S2) | 16 min (S3) |

©Allen Lim

Figure 1. A representative breakdown of intensity zones determined using lactate threshold and VO2max.



Dr. Allen Lim is an exercise physiologist and coach with Thrive Health Fitness Medicine. He received his doctorate at the University of Colorado at Boulder where his research focused on

©Allen Lim

health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: Taking It Nice And Easy Into The Mountains

Floyd remains physically and mentally on track despite weather conditions

By Allen Lim PhD

Stage 6:

Route: Troyes to Nancy
Type: Flat to Rolling Road Race
Distance: 199 km
Elevation Change: 93 meter or 300 feet gain (Average grade = 0.05%)
Total Feet Climbed: 824 meters or 2,704 feet
Average Elevation: 240 ± 88 meters of 788 ± 288 feet above sea level
Minimum Elevation: 111 meters or 364 feet
Maximum Elevation: 398 meters or 1,306 feet/
Weather: 55 to 60°F. Cold, rainy, dangerous and dirty.

Climbs:

1. Cote de Joinville (Cat 4): 1.2 km at 4.5% at 79 km.
2. Cote de Brouthieres (Cat 4): 1.8 km at 4.8% at 102.2 km.
3. Cote de Montigny(Cat 4): 1.4 km at 5.2% at 139.6 km.
4. Cote de Maron (Cat 4): 3.2 km at 5.2% at 182.3 km.

At a Glance:

Stage Placing: 42th
GC Placing: 20th
Time: 04:12:52 (Same time as winner Lorenzo Bernucci)
Average Speed: 47.2 km/hr or 29.4 mph
Average Power: 233 Watts
Total Work: 3523 Kjoules or almost exactly 3523 Kcals burned for Floyd
Stress to Strain Index: 1.07

Predicted Average Power at Front: 444 Watts
% Savings due to Drafting: 52%

Slip and Slide:

Today's stage started out dry and overcast but ended cold, wet and dirty. Of course, in conditions like today there are bound to be crashes. As I was driving through the tight and narrow roads on the course today, I was glad that I wasn't out there riding. Luckily, all the Phonak riders avoided the crash and outside of Floyd getting a flat tire about halfway through the race, the day was actually pretty uneventful. Overall, Floyd thought the conditions made the race a little more difficult but still rated it a 5 on a 1-10 perceived exertion scale. Today might have been the highest average power output (233 watts) of the road stages so far, but it was still only a moderate effort. It looks like we are ramping in nice and easy into the mountains and so far Floyd is physically and mentally right on track.

Calculating Energy Expenditure while Racing:

Part of keeping on track during these early stages and throughout the Tour is proper nutrition and consuming enough calories to maintain weight and energy. This is where the CycleOps Power Tap and our ability to directly measure the actual power output throughout the race brings us a tremendous level of precision and insight.

You might notice that amongst all of the power data, I report how much work is done in a unit called Kjoules. Today's ride was 3,523 Kjoules. Like a Calorie or Kilocalorie, a Kjoule is just another way to represent energy. Because it takes energy to do things different things like riding a bicycle or heating a house, there are also different ways to represent or measure energy. The most common way to measure energy is thermally as the amount of heat something gives off when it's burning. That's how we measure how much energy is in food. Someone literally puts that donut in a pressurized chamber, blows it up, and then measures how much heat it gives off with a thermometer. A calorie is just the amount of heat it takes to raise the temperature of 1 gram of water 1 degree Celsius. So when they say that donut has way too many calories, they know what they're talking about.

Another way to represent energy is mechanically as the amount of force resisting movement over a certain distance. If you've ever tried moving furniture, then you've got a good picture of mechanical work and energy. In the same way that a thermometer measures thermal energy, the CycleOps Power Tap measures mechanical energy. The basic question, however, is this: What's the relationship between Kjoules and Kcals? In other words, how many donuts do I have to burn to move all that furniture or pedal my bike?

Calculating Energy Expenditure while Racing:

Part of keeping on track during these early stages and throughout the Tour is proper nutrition and consuming enough calories to maintain weight and energy. This is where the CycleOps Power Tap and our ability to directly measure the actual power output throughout the race brings us a tremendous level of precision and insight.

You might notice that amongst all of the power data, I report how much work is done in a unit called Kjoules. Today's ride was 3,523 Kjoules. Like a Calorie or Kilocalorie, a Kjoule is just another way to represent energy. Because it takes energy to do things different things like riding a bicycle or heating a house, there are also different ways to represent or measure energy. The most common way to measure energy is thermally as the amount of heat something gives off when it's burning. That's how we measure how much energy is in food. Someone literally puts that donut in a pressurized chamber, blows it up, and then measures how much heat it gives off with a thermometer. A calorie is just the amount of heat it takes to raise the temperature of 1 gram of water 1 degree Celsius. So when they say that donut has way too many calories, they know what they're talking about.

Another way to represent energy is mechanically as the amount of force resisting movement over a certain distance. If you've ever tried moving furniture, then you've got a good picture of mechanical work and energy. In the same way that a thermometer measures thermal energy, the CycleOps Power Tap measures mechanical energy. The basic question, however, is this: What's the relationship between Kjoules and Kcals? In other words, how many donuts do I have to burn to move all that furniture or pedal my bike?

The simple answer is this. 1 Kcal is equal to roughly 4 Kjoules (4.186). So if you do 1000 Kjoules on the bicycle, you've really transferred about 250 Kcals of energy to the rear hub. But does that mean you've only burned 250 Kcals worth of food? The answer is no. Remember, that in **yesterday's article**, I talked a little bit about how a cyclist's efficiency is an important determinant of their ability to produce power. As a reminder, the average cyclist is only about 22% efficient, meaning for every 100 Calories or Kcals they eat, only 22 of those Kcals gets transferred to make the bike move. The rest mostly gets wasted as excess heat. That means if you burn 1000 Kcals of food while riding a bicycle, only about 220 gets transferred to the hub to make the bicycle move. So by a quirk of nature, 1000 Kjoules measured by the Power Tap is equal to just over 1000 Kcals burned by your body. For an average U.S. pro the conversion of Kjoules to Kcals works out to be about 1.1 Kcals for every Kjoule (Table 2). For Floyd the conversion while he's in peak form at the Tour is equal to exactly 1.0. In today's race, doing 3,523 Kjoules means he burned 3,523 Kcals. And this is where the fun begins--getting all those calories replaced so that he can do it all again tomorrow.

Fast Food: For Here and To Go

Everyone always asks me what these guys do in their spare time. Two answers: Eat and Sleep. In addition, to the 3,500 Kcals that Floyd burned in today's race, he also has to consume calories to maintain his already high resting metabolic rate. Those extra calories work out to be 3,000 to 3,500 Kcals. Adding it up and at the end of the day he'll have consumed about 7000 Kcals or 3 to 4 times more than what a normal person needs to eat. Here's a little breakdown of how all that food goes down.

Breakfast:

Like most people, every rider has the basic foods they always eat. In Floyd's case, it's coffee. Whether he was riding in the Tour or hanging out at home he'll always have lots of coffee. In addition to that coffee, breakfast always starts out with 2 to 3 bowls of cereal topped with some muesli, yogurt, and soy milk. 3 maybe 4 eggs and a big plate of pasta follow that. Breakfast or any large meal is eaten at least 3 hours before the race starts so that there is enough time to digest. Those pre-race meals are also filled with foods that have more protein and complex carbohydrates rather than simple sugars - foods that have what we call a low "glycemic index." That is, they're foods that don't spike their blood sugar too much so that we avoid any sugar highs and lows.

Pre-Race:

The riders will typically have a simple snack just before they race -- maybe an energy bar or a few pastries. They're careful not to eat too much within an hour of the start, otherwise, they have problems with digestion and run the risk of getting a sugar low when the race starts. Once they start riding and warming up, however, they'll almost always drink a whole bottle of energy drink.

Race:

During the race, eating is really difficult. Most of the food eaten on the bicycle is the exact opposite of what's eaten at breakfast. They're high glycemic index foods. Foods that bring their blood sugar up really rapidly like candy bars, gels, sweet pastries, Coke, and of course, lots of carbohydrate/electrolyte drinks similar to Gatorade. Because there isn't much blood going to the gut during hard exercise, the riders will try to eat whenever there's a lull in the race to help digestion. They also get most of their calories in during the bike in liquid form, which is easier to absorb. In fact, by the end of the day, almost 70% of all the carbohydrates they take in will be in simple sugars and liquids. If they tried to eat all those calories in solid form two things would happen. The first is that their stomachs just wouldn't be able to handle all that mass and wouldn't digest it all or they'd lose their appetites and lose weight. The second is that if they did get it all in, they'd produce so much fecal matter they probably wouldn't be able to ride the bike the next day.

In the feedbags that are given to the riders at each feed zone and from the cars, there are 3 Power Gels, 2 candy bars, 1 energy bar, 1 small can of coke, 1 bottle of a carbohydrate/electrolyte drink, and 3 pastries wrapped in aluminum foil. Today Floyd went through two of those bags and had 4 bottles of energy drink. He didn't use any of the gels, and so by our calculations he ate about 1600 Kcals on the bike today or about 400 Kcals an hour. In the longer harder days, eating about 1/3rd to 1/2 of what he burns each hour is our basic strategy to keep him from bonking. That is really hard to do. Not only on your stomach but it's also just physically hard to do. In Floyd's case he's adopted this very interesting technique of simply pouring liquids straight down his throat. He just skips the swallowing process all together. He says that it gives him more time to breathe.

Post Race

This is one of the most important meals of the day if not the most important. Immediately after getting into the bus, the riders clean up and start eating and re-hydrating. Immediately after exercise, muscle is really good at reabsorbing energy from food. This enhanced sensitivity is the highest 30-minutes post exercise and slowly fades within 2 to 3 hours. As a result, the food they eat right after getting off the bikes goes right back into their muscles helping them to refuel more quickly for the next day. The basic strategy is to eat as much as is palatable. Foods will include more carbohydrate/electrolyte drinks with a little added protein powder, a sandwich, some boiled potatoes, pastries, and if possible maybe even a plate of pasta with an egg or two on top and plenty of Parmesan cheese and olive oil. On a day like today where he only burned 3,500 Kcals on the bike it's pretty easy for Floyd to replace all the Kcals he burned on the bike post race, especially since he's eating a lot during the race. However, when we get to the really hard stages where he's likely to burn 5,000 to 6,000 Kcals, replacing all those Kcals immediately after will be tough. One very important tool we use to help is a simple scale. Everywhere the team goes, Freddy Viaene, the head soigneur brings the scale. He has the riders weigh themselves every morning and then immediately after they get off the bikes. After a ride, there is a significant amount of fluid loss that is reflected directly in a loss in weight. In the short term, every kilogram of weight lost is equal to 1 kilogram of fluid that needs to be replaced. In the long term, over the course of the race, all the riders will lose a little bit of weight, but if they lose too much too fast, the scale alerts them and they focus on getting more food in.

Dinner:

By the time dinner comes around, the riders are back to normal food. Except that instead of appetizers, they'll almost always start with two huge plates of pasta with red sauce and Parmesan cheese or olive oil and Parmesan cheese, both normally topped with fried egg whites. Then it's on to an extra large salad. And then finally, they'll have a normal meal, like your average human that will consist of some vegetables, rice or potatoes, and some chicken, fish, or steak.

Overall Breakdown:

6,000 to 10,000 Kcals consumed each day or 30 to 50 Krispy Cr? Donuts.

65% Carbohydrate - Over half coming in simple sugars and in liquid form.

20% Protein - Much of it from protein powder, egg whites, and 1 or two servings of animal meat for a total of 1.5 to 2 grams of protein per kilogram of body weight (Average team rider is 70 kg, so that's about 100 to 140 grams of protein. There are 10 grams of protein in 1 egg.)

15% Fat - Olive oil, animal fat, butter, ice cream, and everything tasty.

Table 1. Floyd's power data for stage 6. Beyond the higher average power output, the data looks fairly similar to the other flat stages, except for a bit more time spent at LT and above intensities. This is probably directly related to the greater climbing, more undulating terrain, and cold weather that likely motivated the riders to keep pressure on the pedals.

Table 2. The conversion of Kjoules to Kcals depends on a person's gross mechanical efficiency (GME) or the percent of food energy a person is able to convert to actual mechanical work. For most individuals GME is equal to 22%. While the table above describes this relationship relative to rider experience, the GME is not necessarily dependent on a rider's experience and can be affected by a number of factors like training state, biomechanics, temperature, and the type of ride (e.g., intervals vs. steady). Thus, the number of Kcals burned for a given

| | Stage 6 | Tour Average | Min | Max |
|------------------------------|-------------|----------------------|-------------|-------------|
| Basic Stats | | | | |
| Burial (hrs:min) | 4:32 | 4:06 ± 0:23 | 3:46 (S3) | 4:36 (S3) |
| Distance (miles) | 123 | 120 ± 9.1 | 113 (S5) | 132 (S3) |
| Average Speed (mph) | 29.4 | 29.3 ± 0.61 | 28.6 (S3) | 30.1 (S5) |
| Perception of Effort (1-10) | 5 | 4.5 ± 0.6 | 4 (S2, S3) | 5 (S6, S7) |
| Average Cadence (RPM) | 81 | 86.5 ± 4 | 81 (S6) | 90 (S2) |
| Average Power (Watts) | 233 | 221 ± 11 | 206 (S2) | 233 (S6) |
| Work from Power (Kjoules) | 3523 | 3276 ± 425 | 2855 (S2) | 3743 (S3) |
| Work from RPE (Kjoules) | 3780 | 3642 ± 382 | 2772 (S2) | 3780 (S6) |
| Stress to Strain Index | 1.07 | 1.02 ± 0.11 | 0.88 (S3) | 1.14 (S5) |
| Peak Power | | | | |
| 5 sec (Watts) | 760 | 829 ± 76 | 760 (S6) | 935 (S3) |
| 30 sec (Watts) | 582 | 527 ± 48 | 465 (S2) | 582 (S6) |
| 1 min (Watts) | 441 | 413 ± 30 | 403 (S2) | 473 (S5) |
| 5 min (Watts) | 366 | 347 ± 34 | 308 (S2) | 383 (S3) |
| 30 min (Watts) | 268 | 273 ± 29 | 249 (S2) | 315 (S3) |
| 1 hour (Watts) | 243 | 250 ± 20 | 231 (S2) | 278 (S3) |
| 2 hours (Watts) | 237 | 236 ± 10 | 237 (S2) | 249 (S3) |
| Zone Distribution | | | | |
| Zero Watts (% or min) | 12.7% or 32 | 13 ± 1% or 32 ± 4 | 29 min (S3) | 37 min (S2) |
| Recovery (% or min) | 27.2% or 73 | 29 ± 2% or 72 ± 7 | 65 min (S5) | 80 min (S3) |
| Endurance (% or min) | 33.0% or 83 | 33 ± 2% or 82 ± 12 | 72 min (S2) | 99 min (S3) |
| Lactate Threshold (% or min) | 9.0% or 23 | 8 ± 0.7% or 20 ± 3 | 17 min (S2) | 23 (S3) |
| Race Pace (% or min) | 6.8% or 15 | 6 ± 1% or 15 ± 2.7 | 12 min (S2) | 18 min (S3) |
| Max (% or min) | 4.8% or 12 | 4 ± 0.6% or 10 ± 2 | 8 min (S2) | 12 min (S3) |
| Supra-Max (% or min) | 6.5% or 16 | 6 ± 0.5% or 15 ± 1.9 | 13 min (S2) | 16 min (S3) |

©Allen Lim

number of Kjoules can vary from a high of 1.33 Kcals per Kjoule to a low of 0.92 Kcals per Kjoule depending on the individual and condition. While the table above describes this relationship relative to rider experience, the GME is not necessarily dependent on a rider's experience and can be affected by a number of factors

| Gross Mechanical Efficiency | Correction Factor Kjoules to Kcals | Rider Experience |
|-----------------------------|------------------------------------|------------------|
| 18% | 1.33 | Novice |
| 19% | 1.26 | |
| 20% | 1.19 | Amateur |
| 21% | 1.14 | |
| 22% | 1.09 | U.S. Pro |
| 23% | 1.04 | |
| 24% | 1.00 | Grand Tour Pro |
| 25% | 0.96 | |
| 26% | 0.92 | |

1 A "Calorie" spelled with a capital "C" is actually equal to 1000 "calories" spelled with a lowercase "c." Likewise, 1 Kilocalorie or Kcal is also equal to 1000 calories. By convention on U.S. food labels 1 Kilocalorie is labeled as 1 Calorie, not as 1000 calories. I

©Allen Lim

guess the powers that be didn't want us to think we were eating hundreds of thousands of calories each day even though in actuality we are.

Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: A Week Of Consistency

First week of racing is a mini-taper to the mountains

By Allen Lim PhD

CycleOps

Stage 7: Friday, July 8th

Route: Luneville to Karlsruhe

Type: Mostly flat road race with 1 cat 4 and 1 cat 3 climb

Distance: 228.5 km

Elevation Change: - 114 meter or - 368 feet drop (Average grade = - 0.05%)

Total Feet Climbed: 618 meters or 2,028 feet

Average Elevation: 238 +/- 124 meters or 781 +/- 405 feet above sea level

Minimum Elevation: 118 meters or 387 feet

Maximum Elevation: 637 meters or 2,090 feet

Weather: 57 to 62° F. Cool with sporadic rain

Climbs:

1. Col de la Chipotte (Cat 4): 1.9 km at 4.3% at 45 km.

2. Cote de Brouthieres (Cat 3): 3.5 km at 5.0% at 102.2 km.

At a Glance:

Stage Placing: 85th

GC Placing: 18th

Time: 05:03:45 (Same time as winner Robbie McEwen)

Average Speed: 47.2 km/hr or 29.4 mph

Average Power: 225 Watts

Total Work: 4,101 Kjoules or almost exactly 4,400 Kcals burned for Floyd

Stress to Strain Index:

Predicted Average Power at Front: 395 Watts

% Savings due to Drafting: 44%

Recap: Wet is Good

After every race, I wait for Floyd at the team bus where I get a minute or two to grab the Power Tap computer off the bike and get an assessment of how the day went. Then I'm off to the press center to crunch the numbers and get the power feed sent while Floyd is rushed to the hotel to get cleaned up and recover. That means we don't usually catch up until later in the evening after I've sent the data. So I thought I'd start again by sharing some of Floyd's thoughts from yesterday.

Most of those thoughts had to do with the rain. It may have been a factor for many of the riders yesterday, but for Floyd...well, he actually enjoyed himself. Not that he likes riding in the rain. Just that suffering in the rain is a relative thing and it doesn't bother him as much as it bothers many of the other riders. In particular, on flat cold stages like yesterday and today it's normally the smaller climbing specialists who are most negatively affected.



©Allen Lim

That's because a person's surface area doesn't change proportional with their body weight. A pure climber might be 10 to 20 lbs lighter than the average rider but will only have a slightly smaller surface area. As a result, they tend to get blown around a lot more on the flats. It's like having two almost equally sized kites flying in the wind, with one of them held down by significantly less weight. In addition, the more surface area you have the more area you have to lose body heat. Since the smaller guys have less body mass and produce less absolute power, they don't generate as much heat for a given body area as the larger guys do and end up having a harder time staying warm in the cold and wet rain. When things start going uphill and the temperature rises, however, their smaller body size will be a major advantage since they'll have a better power to weight ratio than the bigger riders and since, relatively speaking, they'll be able to stay a bit cooler. Thus, from our perspective it's probably a good thing that we've got poor weather conditions now during the fast and flat stage because it ends up hurting the small climbers a lot more than it hurts Floyd.

Given that he doesn't mind riding in the rain, I asked Floyd if he had any particular strategies for dealing with the wet weather. Besides just sucking it up and dealing, here are a few he relayed that I thought were interesting.

Remember that in dry conditions, if Floyd is trying to conserve, he'll move to the front at the base of a climb and then drift back over the course of the climb. In wet conditions, Floyd will do the opposite. Instead of drifting back on the climb, he'll actually do a little more work and try and get to the front by the top of the climb. That way when he starts the descent he gets a good view of what's to come. Then over the course of the descent, he'll actually ride slower than the other riders and drift towards the back. Not only is he being conservative, he's also letting riders who are going faster than him run the risk of crashing on any potential oil patches or slick sections of road paint. In a lot of cases, those crashing riders simply point out where the bad sections of road are and with Floyd drifting back he's got more time to react. Essentially, by getting to the top of the climb first, he can ride slower on the descent than others while still keeping in contact with the peloton.

Another trick; he rubs his fingers between his glasses when they get covered in water. The oil on his fingers actually helps to bead the water off the lenses. The effect only lasts for a minute or two, but it's better than not seeing anything for those few minutes. Also, if it's going to rain, he makes sure that he's got a clean or relatively new helmet. If he's wearing an old and dirty helmet, then in the first 5 to 10 minutes of a heavy rain, the accumulated salt in the pads of a dirty helmet will run out and sting the eyes. With a clean helmet, this doesn't happen because there's not that much salt in the pads. Finally, he smiles. When riders get really stressed out in the pack, which is common in the rain, you'll notice that their faces tense up. By actually relaxing his face, he feels that it relaxes the rest of his body and keeps him less tense. Robbie Hunter, Floyd's roommate here at the Tour, however, was quick to point out that if he ever caught Floyd smiling at him in the rain, he'd clobber him.

Into Germany:

With dark clouds over head at the start, I actually felt okay about the possibility of it dumping rain all day. By the end of the day, it actually cleared up and if any of you back home are getting bored of these flat stages, then you'll be upset to learn that the only thing different about Floyd's data today was that the race was longer. That extra length, however, does change a few things. At a similar average power output of 225 watts, a longer duration means Floyd did more work (4,101 Kjoules) and of course burned more Kcals (Just over 4,400 Kcals).

My Bad:

If you've just noticed that today's value for Kcals isn't exactly the same as the number of Kjoules then you actually caught me making a mistake in yesterday's article that Floyd pointed out to me last night. On cold days, we know that Floyd is not quite as efficient. Thus, the conversion of Kjoules to Kcals isn't quite 1 to 1. It's closer to 1 to 1.1, or 10% more than the number of Kjoules done. Floyd also wanted me to point out that while he does eat a lot of food on the bike during the Tour, when he's training and trying to make weight for the Tour he doesn't eat nearly as much. For the average person trying to lose weight by exercising on the bicycle, it's a good reminder that that bottle of Gatorade and Power Bar is probably better left at home.

In any case, today's race was significant only in that Floyd burned more calories today than any other stage to date and that because of the longer duration, he also spent more absolute time in each different intensity zone. Otherwise, we are both really surprised by how consistent these opening flat road stages have been. It's been like the movie Groundhog Day and we're both anxious to either get the girl or get to the mountains.

On Track to Better Training Strategies:

Still, tracking what the first week in the Tour is all about has been truly insightful. It's made us realize that it is possible to use the first week of the Tour as a bit of a mini-taper. Beyond the shear stress of being at this event, Floyd has been able to really conserve and hide. Understanding this first week is also giving us a reference point for how we might design the week leading into hard training periods. In the past, I've found that it's really hard to get an athlete to train all out for more than two weeks at a time before they crack. Thus, it's been hard for me to conceive how someone could get through racing a three-week stage race like the Tour all out. I guess what we're confirming is that it's not possible. Everyday we move along, the power data we collect will become more and more valuable. That's because until you truly understand the actual physical demands of an event, there's no way that you can design a really optimal training program. To that end, tomorrow, I'll take some time and talk about the very basic training principles we use to design Floyd's training and how the power data actually helps bring those basic principles to life.

Table 1. Floyd's power data for stage 7. The major difference between this stage and the previous flat stages was the distance. The riders covered the most distance today giving stage 7 the highest amount of work and most absolute time in different intensity zones. Otherwise, everything else looks exactly like the other stages.

| | Stage 7 | Tour Average | Min | Max |
|-------------------|------------------------------|--------------|----------------------|-------------|
| Basic Stats | Duration (hrs:min) | 5:04 | 4:18 ± 0:12 | 5:46 (S5) |
| | Distance (miles) | 142 | 125 ± 12 | 143 (S5) |
| | Average Speed (mph) | 28 | 29.1 ± 0.8 | 30.1 (S5) |
| | Perception of Effort (1-10) | 4.5 | 4.3 ± 0.5 | 5 (S6, S7) |
| | Average Cadence (RPM) | 88 | 86.8 ± 4 | 90 (S2) |
| | Average Power (Watts) | 225 | 222 ± 10 | 233 (S6) |
| | Work from Power (Kjoules) | 4101 | 3441 ± 520 | 4101 (S7) |
| Peak Power | Work from RPE (Kjoules) | 4101 | 3471 ± 503 | 2772 (S2) |
| | Stress to Strain Index | 1 | 1.01 ± 0.10 | 1.14 (S5) |
| | 5 sec (Watts) | 890 | 842 ± 71 | 760 (S6) |
| | 30 sec (Watts) | 540 | 530 ± 42 | 465 (S2) |
| | 1 min (Watts) | 422 | 439 ± 28 | 403 (S2) |
| | 5 min (Watts) | 356 | 349 ± 30 | 308 (S2) |
| | 30 min (Watts) | 264 | 271 ± 26 | 249 (S2) |
| Zone Distribution | 1 hour (Watts) | 255 | 251 ± 17 | 231 (S2) |
| | 2 hours (Watts) | 235 | 236 ± 8 | 227 (S2) |
| | Zero Watts (% or min) | 13.5% or 41 | 15 ± 2% or 34 ± 5 | 20 min (S3) |
| | Recovery (% or min) | 28.4% or 86 | 29 ± 2% or 75 ± 9 | 65 min (S5) |
| | Endurance (% or min) | 33.0% or 101 | 33 ± 2% or 56 ± 13 | 72 min (S2) |
| | Lactate Threshold (% or min) | 8.2% or 25 | 8 ± 0.6% or 21 ± 3 | 17 min (S2) |
| | Race Pace (% or min) | 6.8% or 21 | 6 ± 0.7% or 15 ± 2.7 | 12 min (S2) |
| Zone Distribution | Max (% or min) | 4.2% or 13 | 4 ± 0.5% or 11 ± 2 | 8 min (S2) |
| | Supra-Max (% or min) | 5.6% or 17 | 5 ± 0.4% or 15 ± 1.9 | 13 min (S2) |

©Allen Lim

Dr. Allen Lim is an exercise physiologist and coach with Thrive Health Fitness Medicine. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising Floyd Landis bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: New Highs

Stage 8: Today's numbers bring us a taste of what's to come

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Pforzheim to Gerardmer

Type: A perfectly flat course sandwiched between 4 category 3 climbs in the first 50 km and 1 category 2 climb at 216 km.

Distance: 231.5 km

Elevation Change: 361 meter or 1,184 feet gain (Average grade = 0.16%)

Total Feet Climbed: 1,912 meters or 6,273 feet

Average Elevation: 317 ± 224 meters of 1045 ± 736 feet above sea level

Minimum Elevation: 137 meters or 449.5 feet

Maximum Elevation: 1139 meters or 3,737 feet

Weather: 55 to 65°F. Cool with sporadic rain once again

Climbs:

1. Cote de Dobel (Cat 3): 5.9 km at 5.9% at 14.5 km.
2. Cote de Bad-Herrenalb (Cat 3): 3.9 km at 4.3% at 27 km.
3. Cote de Nachtigal (Cat 3): 3.8 km at 5.8% at 38.5 km.
4. Cote de Zimmerplatz (Cat 3): 1.9 km at 6.7% at 48 km.
5. Col de la Schlucht (Cat 2): 16.8 km at 4.4% at 216 km

At a Glance:

Stage Placing: 27th

GC Placing: 10th

Time: 05:03:54 (27 seconds down on winner Pieter Weening)

Average Speed: 45.7 km/hr or 28.3 mph

Average Power: 255 Watts

Total Work: 4,650 Kjoules

Stress to Strain Index: 1.18

Predicted Average Power at Front: 416 Watts

% Savings due to Drafting: 39%

Stage 7 Recap: Spirits are Good

Almost all the time that I've spent with Floyd has been during really hard training blocks. So when I'm with him, he's usually beat down and tired, while I'm antsy and wanting to go out. But beat down was exactly how I was feeling yesterday after my first freshman week of Tour madness. When I finally caught up with Floyd at the hotel, it was actually the first time I've ever seen him so relaxed and happy. The role reversal was a good one, as it definitely helped to lift my fatigue. Compared to previous Tours Floyd has done, this first week shift is a great surprise for both of us. Consequently, I don't have much to report from Floyd as I basically just lied on the floor while he watched Tour highlights on T.V. bursting out in laughter and amazement every time they replayed a late race crash - a crash that he has no idea how he got through.

Just a Little Taste:

The last climb today caused a decent separation with only 34 riders making the split over the top of the Col de la Schlucht - a category 2 rated climb that began 216 km into the race and lasted 16.8 km with an average grade of 4.4%. Today, it was this climb and a strong tailwind across the flats leading into it that played the most significant role in the race and in the changes in Floyd's power data.

In today's race, the vast majority of the time Floyd spent at or above his lactate threshold (LT) occurred in the last 2 hours. It was a fast and hard lead up to the climb, with Discovery throttling the pace and not gaining too much for it as all of them were dropped on the final climb. While there seems to be a little bit of a buzz about Lance being isolated, based on Floyd's data, I don't think there's any way that Discovery could have worked that hard and not gotten popped.

In fact, you'll notice that today, Floyd achieved his highest peak power outputs for every single time frame in this year's Tour. All of them occurred in the last two hours, with his peak power for 1-minute, 5-minutes, and 30-minutes each occurring on the final climb, which took the riders just over half an hour to get over. Though Floyd only needed to average 376 watts for the Col de la Schlucht - an intensity that is right at the beginning of his LT, his peak 1-minute power of 524 watts occurred right at the beginning of the climb while his peak 5-minute power of 454 watts occurred over the top of the climb. Over the last 10 minutes of the climb, he averaged 435 watts. If this didn't unload a lot of baggage I'm not sure what would. While plenty of guys can handle a hard surge of 454 watts for 5-minutes, very few can handle that after already riding at close to that intensity the 5 minutes before.

These efforts are so much greater than anything we've seen this week, that the kind of split we saw today makes a lot of sense as does the Discovery absence. In the 30 minutes leading up the climb, the pace was the fastest it was all day. Just to sit in during that time period, Floyd had to average 275 watts. Though these new highs for peak power today are significant, they're still below the best Floyd, and I'm sure his competitors, have produced this year. Thus, this first climb was just a little taste of some real exciting racing to come.

You might also notice in today's data (Table 1) that the finishing time was almost exactly the same as yesterday. I thought that was weird. At almost the same average speed with a little more distance and close to 4000 feet more climbing than yesterday, the race today was definitely a notch up on previous days. Likewise, Floyd gave it his highest rating of any road stage so far lifting the day's stress to strain index to 1.18. With a higher average power and just over 5 hours of racing, Floyd burned more calories today (? 4,900 kcals) than any other stage and I'm sure everyone will be taking an extra serving at dinner tonight. Of note, the strong tail wind and fast speed resulted in Floyd spending the least amount of time not pedaling in any stage yet. Only 26 minutes of coasting for the longest stage this week, compared to almost 41 minutes in yesterday's stage.

One last thing I'd like to point out is that in today's race, Floyd rode a total of 1 hour and 48 minutes at or above his lactate threshold. These are intensity ranges that

are highly dependent on carbohydrate. The average professional cyclist can hold anywhere from 1.5 to 2.5 hours worth of carbohydrate in the form of glycogen in their legs. Once a cyclist is out of glycogen, they'll bonk unless they ingest more carbohydrate. Because, carbohydrate is also used at intensities below the lactate threshold, it is absolutely critical that the riders eat plenty of food during stages like today. It's a great reminder to Floyd that even though he's been able to loaf it a bit this week, the days to come will be orders of magnitude more difficult and that right now, the power data is just a strong reminder to stay focused on the basics.

After this race, however, this data will be the most important intelligence we'll have for designing future training programs for the Tour. In that sense, the real reason we're collecting the data here at the Tour is because it's the best investment we can make for tomorrow. The fact is that a power meter doesn't change the essential nature of training, it simply allows us to actually apply the very basic training principles athletes and coaches have been theorizing about for years.

Basic Training Principles Have Not Changed:

Despite all of the sophisticated technology and science at our disposal today, the type of common sense advice that your parents would give you about life when you were a kid are the same for effective training. Listen to yourself, do your homework, everything takes time, if you're tired then rest, and if you're not then get out of the house. Translated in more academic terms, the fundamentals of training are 1) Specificity, 2) Individuality, 3) Progression, and 4) Periodization.

Specificity:

When I was in school, I loved history but hated math. The problem was, doing my history homework and avoiding the math, never helped me in math class. The same is true with training. If you want to be a good bike racer, you need to race bikes. If you want to win the Tour de France, you need to know what happens in the Tour de France and train specifically for those demands.

As terribly fundamental as this concept may be, in endurance sports where intensity has historically been measured with heart rate or perceived exertion, measuring the specific demands of any given event remained an un-definitive often vague process. It was like knowing that the oven needed to be hot to bake a loaf of bread but never being able to actually measure the temperature of the oven. Instead, you'd stick a loaf of dough in and sometimes it'd come out doughy, sometimes burnt, and only occasionally just right. Through continual trial and error and years of experience, a good baker would eventually figure it out, as have generations of athletes. But imagine speeding up that process with simple objective tools.

In that sense, the power meter has done to training what the thermometer has done for baking and the field of science in general. In fact, if you think about it, the thermometer, which simply measures thermal energy, is perhaps one of our most important scientific inventions. It was the one tool that brought us out of the dark ages as almost every chemical and physical process is dependent on knowing temperature. Thus, knowing temperature spawned a golden age of scientific experiments and discoveries.

Like the thermometer, the power meter also measures energy - mechanical energy - the energy of movement - sometimes play, sometimes toil. So instead of guessing how hard it is to drag that suitcase across the room, you can put a number to it. Just like you can put an objective, reliable, and repeatable number to how hot or cold you feel with temperature, measuring power or watts with Floyd allows us to put objective, reliable, and repeatable numbers to how hard or easy each day in the Tour is. By doing so, we know very specific things. We know exactly how hard a finishing climb might be after 4 hours of racing. We know how many calories he burns after a week of hiding in the peleton. We know that the Tour is the hardest race in the entire world. But now we have the specific temperature or wattage that we have to set his training at to make sure he's better prepared than the next guy.

Individuality:

Although we can now definitively quantify the absolute power and energy required to compete in the Tour, it doesn't mean that anyone can just do that training and be ready for the Tour. One of the reasons we feel comfortable about sharing Floyd's power data is because we know it is specific only to Floyd. Two individuals given exactly the same training will not necessarily adapt in exactly the same way. In addition, every individual here in the Tour is experiencing something different and doing something different every day even though they are all racing in the same pack. So it's important to realize that the information we share on Floyd is by no means representative of the entire pack or individuals in that pack.

With this in mind, you can't follow someone else's recipe for training. You need to take the time to develop your own. Because we have power values and ways to represent Floyd's individual training, that individualized process actually takes less time than mimicking another's approach. To that end, you really do have to just listen to yourself and follow your own instincts making as many mistakes as possible along the way. It's those mistakes that will teach you who you are and how your body responds to work. It's a personal process - that's just the way it is.

Progression:

There's an ancient myth about Milon of Crotona, who in 500 B.C. was considered the world's strongest man. As legend has it, he would begin his training each season by lifting a small calf over his head. As the calf grew, so would Milon's strength, until he was able to lift the full-grown cow. It's exactly this slow, incremental, and patient process that is at the heart of training. In practical terms, by knowing what is required at the Tour de France, we can work backwards in time. So let's say for next year Floyd starts serious prep work in January. At the point in time we can measure how much power he's able to produce for different time frames, do some long test rides and examine how much work he can handle, and then from those references we can plot as progressive of a course to what we expect him to do at the Tour. If he doesn't win this year but we know it would've taken an extra 1 or 2 or 3% more on any given day to win, then we advance that progression so he's well prepared for the next year.

This year, he made tiny steps each week, raising his power by 5 or 10 watts during each training block. While it was never a huge jump, it was the fact that we could always measure those numbers and always had goals set for where we wanted them to be. That's progression in the short term.

In the long-term...well, that started for Floyd when he was a 15-year old kid who would sneak out of the house at 9 at night just to ride his bike. The rest, as they say is history.

Periodization:

The last principle is about balance. You might have often heard people use the word "periodization" when they refer to how they or their coach or their personal trainer sets up their training. While it sounds like a sophisticated term, it's not actually a real word. I know, cause I tried looking it up. What it actually refers to is breaking your training up into different periods.

The concept of periodization was first developed by Russian scientists who found that an athlete's fitness could be optimized if their training was divided into distinct periods of time, each isolating a specific physiological variable or skill important to performance. On a basic level, unique combinations of volume, intensity, and technique characterized different periods of the training season. More importantly, there was the idea that an optimal order existed for developing attributes such as endurance, strength, and speed and that each attribute should be emphasized at different periods over the course of a season or athletic career. A final premise of periodization was that the natural highs and lows associated with training and racing could be controlled with the right combination of work and rest to create specifically timed peaks.

Unfortunately, predicting and manipulating natural patterns, in disciplines ranging from biology to economics, has been the bane of scientists and gurus for hundreds of years. Likewise, the scientific literature examining periodization can be as complex and variable as models for predicting the weather. So here's the long story short. If you're tired, then rest. If you're not then you better train as hard as you can until you're tired. Then you can rest again. We applied this simple principle to Floyd before his Tour prep by setting up 2 to 3 week periods of time (usually with a race in the middle) where he would just attempt to bury himself. When he cracked, he'd get really irritable and then he'd sleep for a day. Just like shaking off a bad hangover. So this year our essential philosophy was to structure big picture ideas in his training (climb more, train harder, etc...) and then on any given day just let him do whatever he could handle. The whole time Floyd would really listen to his own body and go by feel, even if it meant that 4 days before the Tour he was canceling his flight and riding to France. Rather than try to plan everything, we took the opposite approach and tracked everything. What occurred was the discovery of a very natural and consistent oscillation that is specific to Floyd. Now that we know what his periodization pattern is, we are we'll armed for planning his hard training and recovery for next year.

Table 1. Floyd's power data for Stage 8. Brand new max values all across the board.

| | Stage 8 | Tour Average | Min | Max |
|-------------------|------------------------------|--------------|----------------------|-------------|
| Basic Stats | Duration (hrs:min) | 5:04 | 4:25 ± 0:35 | 5:46 (S5) |
| | Distance (miles) | 144 | 128 ± 14 | 173 (S5) |
| | Average Speed (mph) | 28.3 | 28.9 ± 0.8 | 28.1 (S7) |
| | Perception of Effort (1-10) | 6 | 4.8 ± 0.5 | 4 (S2, S3) |
| | Average Cadence (RPM) | 87 | 86.8 ± 3 | 81 (S6) |
| | Average Power (Watts) | 255 | 228 ± 16 | 206 (S2) |
| | Work from Power (KJoules) | 4650 | 3642 ± 678 | 2855 (S2) |
| Peak Power | Work from RPE (KJoules) | 5470 | 3504 ± 932 | 2772 (S2) |
| | Stress to Strain Index | 1.18 | 1.04 ± 0.12 | 0.88 (S3) |
| | 5 sec (Watts) | 965 | 862 ± 51 | 760 (S6) |
| | 30 sec (Watts) | 613 | 544 ± 51 | 465 (S2) |
| | 1 min (Watts) | 513 | 451 ± 39 | 403 (S2) |
| | 5 min (Watts) | 354 | 368 ± 40 | 308 (S2) |
| | 30 min (Watts) | 386 | 290 ± 52 | 249 (S2) |
| Zone Distribution | 1 hour (Watts) | 533 | 265 ± 37 | 231 (S2) |
| | 2 hours (Watts) | 264 | 244 ± 21 | 227 (S2) |
| | Zero Watts (% or min) | 8.6% or 26 | 12.4 ± 3% or 33 ± 5 | 26 min (S8) |
| | Recovery (% or min) | 22.5% or 68 | 28 ± 3% or 74 ± 8 | 65 min (S3) |
| | Endurance (% or min) | 33.7% or 102 | 33 ± 2% or 89 ± 14 | 72 min (S2) |
| | Lactate Threshold (% or min) | 10.6% or 32 | 9 ± 1% or 23 ± 5 | 17 min (S2) |
| | Race Pace (% or min) | 9.6% or 29 | 7 ± 1.5% or 19 ± 6 | 12 min (S2) |
| Zone Distribution | Max (% or min) | 7.2% or 22 | 5 ± 1% or 13 ± 5 | 5 min (S2) |
| | Supra-Max (% or min) | 7.8% or 24 | 6.2 ± 0.9% or 17 ± 4 | 13 min (S2) |

©Allen Lim

Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: Peak Power

Landis finds himself having a consistently paced day

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Stage 9: Sunday, July 10th

Route: Gerardmer to Mulhouse

Type: First mountain stage with 4 category 3 climbs, 1 category 2 climb, and 1 category 1 climb.

Distance: 171 km

Elevation Change: -421 meters or -1,382 feet loss (Average pt to pt grade = - 0.25%)

Total Feet Climbed: 2,552 meters or 8,373 feet

Average Elevation: 637 ± 271 meters of 2,091 ± 890 feet above sea level

Minimum Elevation: 245 meters or 804 feet

Maximum Elevation: 1338 meters or 4,390 feet

Weather: 65° to 70°F. Cool...perfect riding weather.

Climbs:

1. Col de Grosse Pierre (Cat 3): 3.1 km at 6.4% beginning at 6.4 km point.
2. Col des Feignes (Cat 3): 9 km at 2.9% at 22 km point.
3. Col de Bramont (Cat 3): 3.4 km at 6.5% at 33.5 km point.
4. Le Grand Ballon (Cat 2): 21.9 km at 3.6% at 64 km point (High point of stage).
5. Col de Bussang (Cat 3): 6.2 km at 4.5% at 98 km point.
6. Le Ballon d'Alsace (Cat 1): 9.1 km at 6.8% at 115 km point.

At a Glance:

Stage Placing: 74th

GC Placing: 12th

Time: 04:14:24 (6 min & 4 seconds down on winner Michael Rasmussen)

Average Speed: 40.3 km/hr or 25.0 mph

Average Power: 259 Watts

Total Work: 3,953 Kjoules

Stress to Strain Index: 1.16

Predicted Average Power at For Floyd Alone: 350 Watts

% Savings due to Drafting: 26%

Stage 8 Recap:

Floyd was chomping at the bit in yesterday's race but was conservative, remaining patient and quiet. He had a good view of all the strong T-mobile attacks and is excited about the real racing that is yet to come. His legs feel good and after tomorrow's rest day we'll hopefully get to see how good. Otherwise, other than the psychological lift gained by others on the last climb, he didn't see yesterday's stage as all that significant. He was quick to mention that doing an effort like they did yesterday is not that big of a deal if you only have to do it once. As we saw, there are probably 30 or so guys who can do that. But, only half those guys can do that twice, and only 1/3rd can do it three times. So when we get into hard consecutive climbs in the Alps and Pyrenees, it will be a completely different race.

Since I drew an analogy in the Stage 8 article between a power meter and the thermometer, I thought I'd quickly add that even though we can objectively measure temperature, everyone has a different perception of how hot or how cold it is on any given day. This is especially true if it's windy or humid. Likewise, depending on the type of course or environment, how steady or unsteady the pace is, and how fresh or tired a person is, their perception of how hard or easy a ride is might change. So to extend the analogy, in the same way, we often use a "wind chill factor" or "heat index" to correct for temperature, we use the "stress to strain index" to correct for how hard Floyd feels the stage was relative to his actual power. When you look at the total work done in the table, think of that as the actual temperature. Then when you look at the total work done from perceived exertion or RPE, think of that as the wind chill factor for the day.

Stage 9, Still Waiting for More:

Today Floyd came in with his highest average power output of the Tour. However, at just over 4 hours of riding, the total work of 3,953 Kjoules he did today is only slightly higher than the Tour average. In that sense, it was definitely more of a consistently paced day, but with not too much energy expended, the stage was not as difficult as it could've been or as hard as it looked on paper. In fact, though today's profile looked much more aggressive than yesterday's, the 8,373 feet of climbing today was only about 2,000 feet more than yesterday. Also, remember that in much of Floyd's pre-Tour training, he would consistently put in 5,000 to 6,000 Kjoule days with 10,000 to 15,000 feet of climbing. So we know he can handle the work.

The question at hand, however, is how repeated accelerations and surges within a race affect a rider's ability to handle a given amount of work. That is, riding steady by yourself, even at the same average power output or total work, is not quite the same as racing where a hard combination of attacks or surges can end your day before it even begins.

To keep an eye on this we pay careful attention to two things in the data table. First, we look at the peak power outputs he achieves, especially for the 5-minute and 30-minute time frames since most serious attacks occur in this time frame. Second, we look at how much time he spends at and above his lactate threshold, as time accumulated in these intensity zones is exponentially harder than time accumulated below. In today's data, you'll notice that Floyd achieved new highs values for his 30-second peak power, his 1-minute peak power, and his 5-minute peak power. Although this new high for 5-minute peak power is a bit of a flag that today there was a serious punch thrown, Floyd's peak power for 10-minutes and for 30-minutes was much lower than yesterday's. In addition, that 5-minute peak occurred on the first climb of the day, when riders are fresh and probably antsy to test their legs. It'd be a different story if that peak occurred late in the race when everyone is already fatigued.

Also, note that in today's stage, Floyd spent 1 hour and 53 minutes at or above his lactate threshold. Though this is only 5 more minutes than he spent at these intensities compared to yesterday, he did achieve new highs for the Tour for time spent at "lactate threshold" and at "race pace."

Based on all of the data, I'd say it's a toss up as to whether today was really any more difficult than yesterday. So since, the numbers are ambiguous, I'll turn to the man

himself who has just swallowed two pears whole. He's not responding, so I'll just use how I'm feeling relative to how Floyd is looking as a comparison. From my view here in the bus, it looks like Floyd's on a beach in Hawaii, while I'm getting pounded in rush hour traffic. He's showered, clean, smiling and about to get on a plane to Grenoble. I'm sweating, stressed, a bit disoriented and about to drive 5 hours to the same location. So I'd say today was just like the other. The man is good.

Some Thoughts on Peak Power:

Because, I'm often referring to Floyd's peak power data, I'd thought I'd take a little bit of time and officially introduce this very important variable. I'll start by saying that everyone is always asking me what Floyd's maximum power is. As if, he's some sort of racecar and they want a horsepower rating before they haggle with the salesman.

The reality is, there is no such thing as a single maximum power output value for a cyclist. Rather, there are different maximum or peak power values for different time frames. So instead of saying that Floyd's max power is an arbitrary 475 watts, I can say for 5 minutes the highest we've ever seen him hold is 475 watts. That's also why we use the term "peak" instead of "max." When we say "peak," it's a subtle distinction that means the value is the highest we've ever measured to date. When we say, "max," there's the understanding that the value is the highest that has ever and will ever be measured. Since Floyd is still improving, we stick with peak. When he retires, we'll tell you what his max was.

In any case, just like runners can have personal bests or PR's for different distances (the mile, a 10 km run, or a marathon), a cyclist can have personal bests or peaks for different time frames. We measure those bests as the highest wattage a cyclist can for a distinct time period. In our case, we measure the highest power Floyd holds each day for 5 seconds, 30 seconds, 1 minute, 5 minutes, 30 minutes, 1 hour, and 2 hours. The reason why we chose these different time frames is because performing all out for each requires a distinct combination of physiological attributes. As an example, going all out for 5 seconds is really dependent on anaerobic energy sources (i.e., energy production without the use of oxygen), whereas going all out for 2 hours is completely dependent on aerobic energy sources (i.e., energy production exclusively through the use of oxygen). In addition, an all out effort for 5 minutes is very close to an athlete's maximal aerobic capacity or VO2 max while an all out effort for 30 minutes to 1 hour is very close to an athlete's lactate threshold.

Since, these different time frames place distinct physiological demands on the body, cyclists who are better at sprinting tend to have better peak power outputs in the 5 second to 1 minute time frame, while cyclists who are better at time trialing or climbing normally have better peak power outputs for the 5 minute to 2 hour time frame. A sprinter here at the Tour might be able to hold 1700 or more watts for 5 seconds while Floyd probably won't crack 1000 watts. On the other hand, Floyd will probably be able to hold over 400 watts for 30 minutes, while a sprinter of equal weight will be lucky to hold over 350 watts.

Regardless of the type of cyclist, however, there is an exponential or cubic like relationship between a given time frame and the power that can be held. In other words, there's a real fast and steep drop in peak power output between 5 seconds and 1 minute, and a smaller drop between 5 minutes and 30 minutes, and a shallow drop between 1 hour and 2 or more hours.

Ultimately, we know what Floyd's all time peaks are for each time frame and here at the Tour we keep a daily check not only on how each day's values compares to the other but how each day's values compare with what we know his best to be. Although we expect him to record new PR's here at this year's Tour, I'll just say that we haven't seen them yet. But I'm sure we will.

For a reference table of peak power values for pro cyclists and your average Jane and Joe, take a look at **tables 2 and 3**.

Table 1. Floyd's power data for Stage 9. A higher average power today, but with less time, the total work was not much higher than the average for the Tour. Overall a more consistent day of work for Floyd with new high peak power outputs at 30 seconds, 1 minute, 5 minutes and 2 hours. For the first climbing day, not quite as hard as yesterday in terms of climbing, but more climbs and still more time at LT and race pace zones.

| | Stage 9 | Tour Average | Min | Max |
|------------------------------|-------------|----------------------|-------------|--------------|
| Basic Stats | | | | |
| Duration (hours:min) | 4:14 | 4:24 ± 0:32 | 3:46 (S5) | 5:04 (S8) |
| Distance (miles) | 106 | 125 ± 15 | 113 (S5) | 144 (S8) |
| Average Speed (mph) | 25.0 | 28.4 ± 1.65 | 25.0 (S9) | 30.1 (S5) |
| Perception of Effort (1-10) | 6 | 4.9 ± 0.8 | 4 (S2, S3) | 6 (S8, S9) |
| Average Cadence (RPM) | 83 | 86.3 ± 3 | 81 (S6) | 90 (S2) |
| Average Power (Watts) | 259 | 232 ± 19 | 208 (S2) | 259 (S9) |
| Work from Power (KJoules) | 3953 | 3687 ± 631 | 2855 (S2) | 4639 (S8) |
| Work from RPK (KJoules) | 4579 | 3915 ± 900 | 2772 (S2) | 5470 (S8) |
| Stress to Strain Index | 1.16 | 1.06 ± 0.11 | 0.88 (S3) | 1.38 (S8) |
| Peak Power | | | | |
| 5 sec (Watts) | 755 | 847 ± 83 | 760 (S6) | 965 (S8) |
| 30 sec (Watts) | 645 | 558 ± 66 | 465 (S2) | 645 (S9) |
| 1 min (Watts) | 555 | 466 ± 55 | 403 (S2) | 555 (S9) |
| 5 min (Watts) | 478 | 382 ± 63 | 308 (S2) | 478 (S9) |
| 30 min (Watts) | 337 | 297 ± 51 | 249 (S2) | 296 (S9) |
| 1 hour (Watts) | 360 | 270 ± 36 | 231 (S2) | 333 (S8) |
| 2 hours (Watts) | 287 | 244 ± 21 | 227 (S2) | 287 (S9) |
| Zone Distribution | | | | |
| Zero Watts (% or min) | 12.5% or 32 | 12.4 ± 2% or 32 ± 5 | 26 min (S8) | 41 min (S7) |
| Recovery (% or min) | 17.2% or 44 | 25 ± 5% or 69 ± 14 | 44 min (S9) | 89 min (S7) |
| Endurance (% or min) | 31.2% or 81 | 53 ± 2% or 88 ± 13 | 72 min (S2) | 102 min (S8) |
| Lactate Threshold (% or min) | 14.0% or 36 | 9 ± 2% or 25 ± 7 | 17 min (S2) | 36 min (S9) |
| Race Pace (% or min) | 12.3% or 31 | 8 ± 2% or 20 ± 7 | 12 min (S2) | 31 min (S9) |
| Max (% or min) | 6.4% or 16 | 5 ± 1% or 13 ± 5 | 8 min (S2) | 22 min (S8) |
| Supra-Max (% or min) | 5.7% or 15 | 6.1 ± 0.8% or 16 ± 4 | 13 min (S2) | 24 min (S8) |

©Allen Lim

Table 2. Peak absolute power output ranges for women and men of different racing categories and for untrained individuals.

| | | Estimated Ranges for Peak Sustainable Power | | | | | | | | | |
|-----------|--------------|---|--------|------|--------|--------|--------|-------|--------|--------|---------|
| | | Absolute Power (Watts) | | | | | | | | | |
| | | 2 hrs | | 1 hr | | 30 min | | 5 min | | 30 sec | |
| Women | Int Pro | 193 | to 222 | 225 | to 252 | 255 | to 270 | 283 | to 310 | 646 | to 760 |
| | Domestic Pro | 179 | to 207 | 210 | to 234 | 237 | to 251 | 260 | to 292 | 570 | to 699 |
| | Cat 1-2 | 167 | to 193 | 196 | to 219 | 222 | to 234 | 246 | to 269 | 515 | to 644 |
| | Cat 3 | 153 | to 176 | 178 | to 200 | 203 | to 214 | 223 | to 246 | 470 | to 587 |
| | Cat 4 | 132 | to 152 | 154 | to 172 | 175 | to 185 | 192 | to 214 | 406 | to 507 |
| Men | Untrained | 113 | to 128 | 130 | to 145 | 147 | to 156 | 160 | to 182 | 342 | to 428 |
| | Int Pro | 275 | to 317 | 321 | to 359 | 364 | to 385 | 394 | to 452 | 846 | to 1057 |
| | Domestic Pro | 252 | to 291 | 295 | to 330 | 334 | to 353 | 372 | to 404 | 777 | to 971 |
| | Cat 1-2 | 235 | to 271 | 275 | to 307 | 311 | to 329 | 346 | to 378 | 724 | to 904 |
| | Cat 3 | 216 | to 249 | 253 | to 283 | 286 | to 303 | 319 | to 346 | 645 | to 831 |
| Untrained | Cat 4 | 182 | to 209 | 212 | to 237 | 240 | to 254 | 266 | to 293 | 539 | to 698 |
| | Untrained | 147 | to 170 | 172 | to 192 | 194 | to 206 | 213 | to 239 | 452 | to 565 |

©Allen Lim for women and men of

different racing categories and for untrained individuals.

| | | Power to Weight (Watts per Kg) | | | | | | | | | |
|-----------|--------------|--------------------------------|---------|------|---------|--------|---------|-------|---------|--------|----------|
| | | 2 hrs | | 1 hr | | 30 min | | 5 min | | 30 sec | |
| Women | Int Pro | 3.21 | to 3.71 | 3.75 | to 4.20 | 4.25 | to 4.50 | 4.71 | to 5.17 | 10.77 | to 12.67 |
| | Domestic Pro | 2.99 | to 3.45 | 3.49 | to 3.99 | 3.95 | to 4.18 | 4.33 | to 4.86 | 9.50 | to 11.65 |
| | Cat 1-2 | 2.79 | to 3.22 | 3.26 | to 3.65 | 3.69 | to 3.91 | 4.10 | to 4.48 | 8.59 | to 10.74 |
| | Cat 3 | 2.54 | to 2.94 | 2.97 | to 3.33 | 3.37 | to 3.56 | 3.72 | to 4.10 | 7.83 | to 9.79 |
| | Cat 4 | 2.20 | to 2.64 | 2.57 | to 2.87 | 2.91 | to 3.08 | 3.19 | to 3.57 | 6.76 | to 8.46 |
| Men | Untrained | 1.85 | to 2.14 | 2.17 | to 2.42 | 2.45 | to 2.59 | 2.66 | to 3.04 | 5.70 | to 7.13 |
| | Int Pro | 3.93 | to 4.53 | 4.59 | to 5.14 | 5.20 | to 5.50 | 5.62 | to 6.46 | 12.09 | to 15.11 |
| | Domestic Pro | 3.61 | to 4.16 | 4.22 | to 4.73 | 4.77 | to 5.05 | 5.22 | to 5.78 | 11.40 | to 13.57 |
| | Cat 1-2 | 3.36 | to 3.88 | 3.93 | to 4.39 | 4.44 | to 4.70 | 4.94 | to 5.40 | 10.34 | to 12.92 |
| | Cat 3 | 3.09 | to 3.56 | 3.61 | to 4.04 | 4.09 | to 4.32 | 4.54 | to 4.94 | 9.50 | to 11.83 |
| Untrained | Cat 4 | 2.59 | to 2.99 | 3.03 | to 3.39 | 3.43 | to 3.63 | 3.80 | to 4.18 | 7.98 | to 9.93 |
| | Untrained | 2.10 | to 2.42 | 2.45 | to 2.75 | 2.78 | to 2.94 | 3.04 | to 3.42 | 6.46 | to 8.08 |

©Allen Lim

Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: How Hard Is That Climb?

Is the Tour's system or rating climbs flawed?

By Allen Lim PhD

Stage 10: Tuesday, July 12th

Route: Grenoble to Courchevel

Type: Mountain top finish with two category 1 climbs.

Distance: 192.5 km

Elevation Change: 1780 meters or 5840 feet gain (Average pt to pt grade = 0.92%)

Total Feet Climbed: 3,293 meters or 10,804 feet

Average Elevation: 801 ? 577 meters of 2,627 ? 1893 feet above sea level

Minimum Elevation: 220 meters or 722 feet

Maximum Elevation: 2000 meters or 6,562 feet

Weather: 60° to 75°F and clear. Another perfect day for riding.

Climbs:

1. Cornet de Roselend (Cat 1): 20.1 km at 6.0% ending at 118 km point.
2. Courchevel (Cat 1): 22.2 km at 6.2% ending at 192.5 km point.

At a Glance:

Stage Placing: 11th (4 min & 16 seconds down on Lance Armstrong)

GC Placing: 10th

Time: 04:52:49 (2 min & 14 seconds down on winner Alejandro Valverde)

Average Speed: 36.5 km/hr or 23.0 mph

Average Power: 268 Watts (Floyd Predicted)

Total Work Estimate: 4,700 Kjoules (Floyd Predicted)

Stress to Strain Index: 1.48

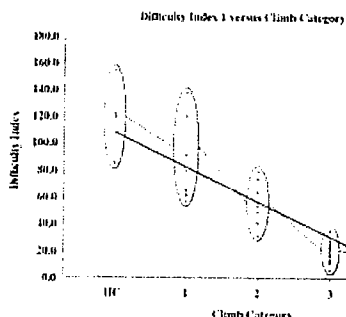
No Excuses from Floyd:

There were a lot of expectations for today's first major mountain stage. In the end, Floyd gave it everything he had, but was not there in the final. Still, he is still amongst many of the favorites and it is still a very long and difficult race to come. As the days get more and more difficult it will be important for him to ride within himself and let his natural ability to recover and his natural endurance bring him up the standings. **My Opinion:**

In some ways, I feel that the ride today wasn't hard enough to suit his strengths. Since it was the first major mountain stage immediately after a rest day, there were definitely more guys who were fresh. That combined with only two major climbs and a rejuvenated Discovery team made it a hard day for everyone. As the days wear on and fatigue mounts across the peloton, I really do believe Floyd will begin to shine. It's been a tough race so far, but adversity is a good thing and I'm looking forward to seeing Floyd shine in the really arduous days to come. I know it's what he really thrives on.

It Won't Happen Again:

I am sorry to report that we don't have any power data to report today. Floyd got a rear flat very early in the stage and in the confusion was not able to get a Power Tap rear, despite there being spares in the car. The mechanics tell me it won't happen again as they know the power data is extremely important to Floyd. In particular, it's really the days that don't go as planned that are the most important to us. It's finding the weaknesses that give you the real foundation for becoming stronger.



©Allen Lim

How the Power Tap Helps Floyd in the Here and Now:

An article or two back, I mentioned that one of the primary reasons why we are collecting all the power data here is to have a basis for future training strategies. That's one of the reasons why not getting today's data is really disappointing.

I was recently asked, however, how the data helps Floyd real time on the bicycle. The major way it helps him is that it gives him instantaneous feedback to help him gauge his efforts and to help remind him to stay within the parameters of a particular strategy we may have set.

Although, Floyd is extremely in tune with his body, that highly developed sense of feel has been developed almost entirely by constantly seeing his power values flashed before him while racing and training. In fact, in the last year, today was the first day, outside of a time trial, that Floyd did not have the power meter. The thing missed most from having it on the bike today, were the constant reminders of where he's at, that in a millisecond help him to calibrate his effort. In the end, it helps to keep him relaxed and focused on his own ride in the middle of all the chaos of racing. The information can help him temper his efforts on attacks or help him establish a more optimal pace for himself on a climb. On the flats, watching the meter was one of the main

ways he is able to conserve so much energy. It nags him to find the sweet spots in the pack and float. In fact, he found that just a little farther back from the nose of the pack he was able to save significantly more energy without compromising his safety. That may explain why many of our predictions for energy saving from drafting hovered around 50% for the flat stages, compared to the 40% that is commonly reported.

Still, in the middle of a race, it's often about just going for it. The numbers aren't there to obsess about or there to dictate the race. They are there for feedback and to help record information in a way that is more objective than our best memory of the race.

How Hard is That Climb?

Because I didn't have any power data to analyze, I took the time to crunch way too many numbers in an attempt to figure out a question that has been bothering me for a really long time now. That question has to do with the Tour de France organization's system for rating all the climbs during the race and what those different categories really mean.

The climbs in the Tour are rated using a 4-category system. In reality, there are 5 categories of climbs. The categories are based on 3 major factors. They are 1) the steepness of the climb, 2) the length of the climb, and 3) the position of that climb along the course. Theoretically, a really steep and long climb occurring late in the race would receive the most difficult rating. A climb can be rated a 1, 2, 3, 4 or Hors Catégorie (HC). The easiest climb is a category 4 rated climb, while a 3, 2, and 1 are incrementally harder. The "Hors Catégorie" is a climb that is so difficult that it is beyond categorization. In my mind they should just add another number to the scale and they should reverse the order so a 1 is easiest, etc. In any case, the climb categorization is then used to determine the value of points given for the best climber or "Polka Dot" jersey competition with more points are given on the harder climbs.

To find out exactly how hard each climb in the Tour is, I actually entered every single category 1 to 4 and out of category climb into a spread sheet and looked at the following factors:

A. The point on the course where a climb as a percentage of the total distance that day.

B. The percent grade of the course.

C. The total distance of the climb.

D. The total feet climbed.

E. The elevation of the climb.

Based on these factors, I figured that if the category system established by the Tour did actually mean something, then by multiplying or adding the percent grade, the % at which the climb occurred, and the length of the climb, I could get a "Difficulty Index" that would actually tell me how hard a given climb is.

What I found, though, was that my system, which actually did take into account the steepness of the climb, the length of the climb, and the position of that climb along the course, was only related to the Tour's 1-4 to HC system 65% of the time. After all that work, that finding made me really angry. So I then decided to be an even bigger nerd and figure out what factors were best related to the Tour's ranking system. I found that if I just took into account the climbs length and steepness, I could predict the actual Tour category 80% of the time. So long story short, the Tour's category system from what I can tell is best related to the length and steepness of the climb and only predicts the length and steepness 80% of the time. In my mind the system is flawed. I shared this information with some of the riders and they laughed at me saying that they could've just told me that the rating system was completely arbitrary. I guess if you are going all out for 3 weeks whether your going up or down has no bearing on how hard you think things are.

Anyway, here are some interesting things I learned about the climbs in of the Tour.

For all 64 categorized climbs:

% Grade:

Average: $5.5 \pm 1.6\%$

Minimum: 2.9%

Maximum: 10.1%

Length of Climb:

Average: 6.8 ± 6.0 Km

Minimum: 0.9 Km

Maximum: 25.4 Km

Elevation Gain:

Average: $1,271 \pm 1,230$ feet

Minimum: 105 feet

Maximum: 5,059 feet

For all 22 Category 4 Climbs:

% Grade:

Average: $4.6 \pm 1.1\%$

Minimum: 2.9%

Maximum: 6.6%

Length of Climb:

Average: 2.7 ± 1.9 km

Minimum: 0.9 km

Maximum: 8.3 km

Elevation Gain:

Average: 363 ± 187 feet

Minimum: 105 feet

Maximum: 791 feet

For all 19 Category 3 Climbs:

% Grade:

Average: $5.3 \pm 1.2\%$

Minimum: 2.9%

Maximum: 7.1%

Length of Climb:

Average: 4.3 ± 2.6 km

Minimum: 1.2 km

Maximum: 11.3 km

Elevation Gain:

Average: 675 ± 262 feet

Minimum: 190 feet

Maximum: 1,168 feet

For all 8 Category 2 Climbs:

% Grade:

Average: 5.6 ± 2.1%

Minimum: 3.6%

Maximum: 10.1%

Length of Climb:

Average: 11.7 ± 5.9 Km

Minimum: 3.1 Km

Maximum: 21.9 Km

Elevation Gain:

Average: 1,829 ± 533 feet

Minimum: 1,017 feet

Maximum: 2,585 feet

For all 10 Category 1 Climbs:**% Grade:**

Average: 7.2 ± 0.8%

Minimum: 6.0%

Maximum: 8.3%

Length of Climb:

Average: 11.6 ± 5.4 Km

Minimum: 7.0 Km

Maximum: 22.2 Km

Elevation Gain:

Average: 2,620 ± 919 feet

Minimum: 1,850 feet

Maximum: 4,492 feet

For all 5 Hors Category Climbs:**% Grade:**

Average: 7.3 ± 0.9%

Minimum: 6.1%

Maximum: 8.3%

Length of Climb:

Average: 17 ± 5.5 Km

Minimum: 10.3 Km

Maximum: 25.4 Km

Elevation Gain:

Average: 3,939 ± 805 feet

Minimum: 2,789 feet

Maximum: 5,059 feet

Hardest climb based on steepness, length, and location on course:

- Today's last climb Courchevel at 6.2% grade for 22.2 Km (2,070 feet to 6,563 feet for a gain of 4,492 feet)

Hardest climb based on just steepness and length:

- Tomorrow's climb up the Col de Madeleine at 6.1% grade for 25.4 Km (1,503 feet to 6,562 feet for a gain of 5,059 feet)

*Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.*

Bicycling

Powerfeed: Consistency Over The Long Haul

Tactics and terrain of stage produce new max values

By Allen Lim PhD

Stage 11: Wednesday, July 13th

Route: Courchevel to Briancon

Type: Mountain stage with two HC category climbs and a category 1 climb.

Distance: 173 km

Elevation Change: -237 meters or -449 feet loss (Average pt to pt grade = - 0.08%)

Total Feet Climbed: 3,846 meters or 12,619 feet (Most climbing to date)

Average Elevation: 1277 \pm 580 meters of 4,191 \pm 1,904 feet above sea level

Minimum Elevation: 458 meters or 1,503 feet

Maximum Elevation: 3,645 meters or 8,678 feet

Weather: 70 to 75°F. Mostly clear skies all day.

Climbs:

1. Col de Madeleine (HC): 25.4 km at 6.1% beginning at 25.4 km point.
2. Col du Telegraphe (Cat1): 12 km at 6.6% at 98 km point.
3. Col de Galibier (HC): 17.5 km at 6.9% at 115.5 km point.

At a Glance:

Stage Placing: 19th

GC Placing: 11th

Time: 04:48:53 (1 min & 15 seconds down on winner Vinokourov)

Average Speed: 35.9 km/hr or 22.3 mph

Average Power: 285 Watts

Total Work: 4,940 Kjoules

Stress to Strain Index: 1.23

Feeling Better:

Today was great for the team with Floyd getting a pretty comfortable ride into Briancon, following wheels as his teammates Santiago Botero and Oscar Pereiro went on the attack on some of the longest and consistently steep climbs of the Tour. In the end Botero was able to stay away with Alexandre Vinokourov but got out sprinted at the line. It was still an exceptional day for Botero who won here in Briancon 5 years ago. He's beginning to show serious form and the team as a whole is feeling good about their options in the stages to come.

Floyd in particular felt a lot better compared to **yesterday's mountain top finish in Courchevel**. He was super excited for his teammates as he rolled into the bus today, raising his arms in victory as if they had won the race. Regardless of the outcome it is always a great feeling to finish a race knowing that the team was able to execute without mishap or mistake.

Today's Power:

The power data from today really reflects the tactical and terrain features of the day. In particular, the race produced a couple of significant new max values. A new high average power of 285 Watts, a new high workload of 4,940 Kjoules, a new high perceived workload of 6,067 Kjoules, a new max power output for 1 hour (359 Watts) and 2 hours (315 Watts), and an almost two fold increase in the time spent at lactate threshold (66 minutes) and race pace (44 minutes).

Table 1

These new highs reflect a more even pace across the day. With Botero and Pereiro on the attack and Discovery setting a constant tempo to keep them in check, the intensity was elevated all day but lacked the same kind of high intensity or supra-maximal surges that we've seen in previous stages. In addition, with some of the longest and consistently graded climbs that we will see in this Tour, the terrain called for consistency over the long haul rather than short attacks.

Though it could be said that **this stage** was the hardest yet, it was hard in a different way. The overall power and work came up, but with average values for time spent in Floyd's max and supra-max zones as well as unremarkable peak power outputs for 1, 5, and 30-minutes it is unlikely that there were too many in the red today - a scenario that is reflected by the larger lead finishing group. The power from today also demonstrates that even though something may look really tough on paper it's how an individual, or the peleton as a whole, chooses to ride it that makes the day easy or difficult. In fact, today Floyd came nowhere close to the peak power outputs for 5, 10, and 30-minutes that he achieved in stage 8 and 9 on climbs that were rated significantly lower than the ones today.

Calculating Power Old School: A Look at "VAM"

Because the grade and pace on today's climbs were so consistent, it is possible to do a quick check of the power numbers using calculations that for some are still

| | Stage II | Tour Average | Min | Max |
|------------------------------|-------------|-----------------------|--------------|--------------|
| Basic Stats | | | | |
| Duration (hrs:min) | 4:48 | 4:30 ± 1:30 | 3:46 (S5) | 5:04 (S8) |
| Distance (miles) | 107 | 121 ± 14 | 107 (S11) | 144 (S8) |
| Average Speed (mph) | 22.5 | 27.1 ± 2.92 | 22.3 (S11) | 25.0 (S9) |
| Perception of Effort (1-10) | 7 | 5.5 ± 1.4 | 4 (S2, S3) | 8 (S10) |
| Average Cadence (RPM) | 81 | 85.0 ± 3.5 | 81 (S6, S11) | 90 (S2) |
| Average Power (Watts) | 285 | 242 ± 26 | 206 (S2) | 285 (S11) |
| Work from Power (KJoules) | 4940 | 3944 ± 748 | 2855 (S2) | 4940 (S11) |
| Work from RPE (KJoules) | 6067 | 4500 ± 1420 | 2772 (S2) | 6067 (S11) |
| Stress to Strain Index | 1.21 | 1.12 ± 0.17 | 0.88 (S5) | 1.48 (S10) |
| Peak Power | | | | |
| 5 sec (Watts) | 757 | 816 ± 84 | 757 (S11) | 963 (S8) |
| 30 sec (Watts) | 553 | 558 ± 56 | 465 (S2) | 645 (S9) |
| 1 min (Watts) | 505 | 471 ± 51 | 403 (S2) | 555 (S9) |
| 5 min (Watts) | 431 | 388 ± 60 | 308 (S2) | 478 (S9) |
| 30 min (Watts) | 363 | 305 ± 53 | 249 (S2) | 386 (S8) |
| 1 hour (Watts) | 359 | 281 ± 46 | 231 (S2) | 359 (S11) |
| 2 hours (Watts) | 315 | 250 ± 32 | 227 (S2) | 315 (S11) |
| Zone Distribution | | | | |
| Zen Watts (% or min) | 9.2 % or 27 | 12.0 ± 2.5% or 32 ± 5 | 26 min (S8) | 41 min (S7) |
| Recovery (% or min) | 12.4% or 36 | 25 ± 0.7% or 63 ± 17 | 36 min (S11) | 86 min (S7) |
| Endurance (% or min) | 38.6% or 85 | 35 ± 1.7% or 58 ± 12 | 72 min (S2) | 102 min (S8) |
| Lactate Threshold (% or min) | 23.0% or 66 | 11 ± 5% or 30 ± 16 | 17 min (S2) | 64 min (S11) |
| Race Pace (% or min) | 15.2% or 44 | 9 ± 3.5% or 23 ± 11 | 12 min (S2) | 44 min (S11) |
| Max (% or min) | 3.5% or 10 | 5 ± 1% or 13 ± 5 | 8 min (S2) | 22 min (S8) |
| Supra-Max (% or min) | 4.3% or 12 | 5.9 ± 1.0% or 16 ± 4 | 12 min (S11) | 21 min (S8) |

©Allen Lim

the norm for estimating a rider's fitness and power.

That technique is often referred to as calculating a rider's "VAM" or Vertical Ascent rate in Meters per hour. It's based on the simple premise that the faster you climb up a hill the more power you have to be producing. Indeed, the physics of riding up a steep climb (? 7% grade) is actually quite simple. Thus, measuring how fast someone moves up a hill is one of the simplest ways to derive power.

Up a steep hill, about 90% of the power a cyclist produces is used to overcome gravitational resistance, which is a function of body and bicycle weight. At the same speed, a heavier cyclist will produce a higher power output than a lighter cyclist on a given climb. That's why when we talk about climbing ability we almost always talk about it in terms of a cyclist's power to weight ratio rather than the absolute power.

For example, to stay with the lead group today on the Galibier, you would've had to produce about 5 to 5.5 Watts per Kilogram of body weight. For an average Tour cyclist who weighs 70 Kg or 154 pounds, that would be 350 to 375 Watts. For a lighter cyclist weighing 60 Kg their power would only have to be 300 to 330 Watts.

In either case, if we know a cyclist's body and bicycle weight, the elevation gain over the course of a hill, and their time up a hill, we can get a decent estimate of their power output. The calculation is as follows:

$$\text{Power (W)} = (\text{Total Mass in Kg} \times 9.8 \times \text{Elevation Gain in Meters}) \div \text{Time in Seconds}$$

As an example, up the Galibier today the riders gained 3,954 feet in elevation or 1,205 meters. Let's say it takes them 45 minutes to get to the top (2,700 seconds) and that the average rider is 70 Kg (154 lbs) with a bike weight of 6.8 Kg (15 lbs). Thus, their power for that time would be equal to:

$$\text{Power} = (76.8 \text{ Kg} \times 9.8 \times 1,205 \text{ Meters}) \div 2,700 \text{ seconds} = 336 \text{ Watts}$$

Remember, however that the power to get up the hill is only about 90% of the total power. The additional 10% is used to overcome a small amount of aerodynamic and rolling resistance. Thus if we add 10% to this value we get a total of 370 Watts to climb the Galibier in 45-minutes.

Since a cyclist's power is just proportional to their rate of ascent, you don't really have to calculate the actual power output to appreciate how powerful a cyclist is. Instead old coaches and directors would just calculate the rate of ascent in meters per hour using altitude watches while following a rider or the pack. So another way to look at climbing effort is to simply say that if you can climb the Galibier in 45 minutes, you would be climbing 1,205 meters in 45 minutes or a VAM of 1607 meters per hour.

Most professional cyclists can achieve a VAM of 1400 to 1500 meters per hour. Really great climbers can climb at a VAM of 1600 to 1700 meters per hour. Finally, there are a select few (Pantani, Mayo, Armstrong) who have broken the holy grail or 4-minute mile of climbing speed - a VAM of 1800 meters per hour which is equal to about 6.3 Watts per Kilogram of body weight up an 8% grade. That's 455 Watts for Lance Armstrong up L'Alp D'Huez last year. Remember, however that the power to get up the hill is only about 90% of the total power. The additional 10% is used to overcome a small amount of aerodynamic and rolling resistance. Thus if we add 10% to this value we get a total of 370 Watts to climb the Galibier in 45-minutes.

Since a cyclist's power is just proportional to their rate of ascent, you don't really have to calculate the actual power output to appreciate how powerful a cyclist is. Instead old coaches and directors would just calculate the rate of ascent in meters per hour using altitude watches while following a rider or the pack. So another way to look at climbing effort is to simply say that if you can climb the Galibier in 45 minutes, you would be climbing 1,205 meters in 45 minutes or a VAM of 1607 meters per hour.

Most professional cyclists can achieve a VAM of 1400 to 1500 meters per hour. Really great climbers can climb at a VAM of 1600 to 1700 meters per hour. Finally, there are a select few (Pantani, Mayo, Armstrong) who have broken the holy grail or 4-minute mile of climbing speed - a VAM of 1800 meters per hour which is equal to about 6.3 Watts per Kilogram of body weight up an 8% grade. That's 455 Watts for Lance Armstrong up L'Alp D'Huez last year. In any case, it's really nice to see that what Floyd actually did is extremely close to what we predict for the terrain and speed. Specifically, on today's final climb, the lead group averaged 20.5 km/hr or 12.7 mph and climbed at a rate of 1413 meters per hour, which is well within the capacity of many riders here in the Tour. This VAM combined with the actual power readings at our disposal is further confirmation for the final group size.

Ultimately, if there's a lesson in all the calculations I just listed out here, it's that you can't just blindly trust the numbers you get from a power meter, nor can you assume that riding conditions are consistent enough that you can always know how hard a cyclist is going just by looking at their speed or VAM. In the end, you need both. A little bit of old school to confirm the new school and a little bit of new school to give props to the old.

For a detailed look at how the percent grade and speed affect absolute power output, power output normalized to weight, VAM, and the % of power used to overcome gravity see **Tables 2, 3, 4, and 5**.

Table 2. Power required for different speeds and grades in a 154 lb rider.

| | | Power Required for a Given Speed (mph) and Grade (%) for a 70 kg (154 lb) Person with a 6.8 kg (15 lb) Bicycle | | | | | | | | | |
|-------------|-----|---|-----|-----|-----|-----|-----|-----|------|------|------|
| Speed (mph) | S | Percent Grade | | | | | | | | | |
| | | 1% | 2% | 3% | 4% | 5% | 6% | 7% | 8% | 9% | 10% |
| 7.5 | 27 | 11 | 61 | 78 | 95 | 112 | 129 | 145 | 162 | 179 | 197 |
| 10 | 45 | 50 | 95 | 120 | 146 | 171 | 196 | 221 | 247 | 272 | 297 |
| 12.5 | 67 | 100 | 134 | 168 | 202 | 235 | 269 | 303 | 337 | 370 | 404 |
| 15 | 94 | 136 | 178 | 220 | 262 | 304 | 346 | 388 | 430 | 472 | 514 |
| 17.5 | 130 | 180 | 231 | 281 | 332 | 382 | 433 | 483 | 534 | 584 | 634 |
| 20 | 174 | 232 | 292 | 351 | 410 | 469 | 529 | 588 | 647 | 706 | 765 |
| 22.5 | 227 | 295 | 362 | 429 | 496 | 563 | 631 | 698 | 765 | 832 | 899 |
| 25 | 297 | 373 | 449 | 525 | 601 | 677 | 753 | 829 | 906 | 982 | 1058 |
| | 379 | 464 | 548 | 633 | 717 | 802 | 887 | 971 | 1056 | 1140 | 1225 |

©Allen Lim

Table 3. Power to weights (Watts per Kg of body weight) required for different speeds and grades. Data is based on a 154 lb rider with a 15 lb bicycle. Generally speaking, the cyclist with the highest power to weight ratio can maintain the highest climbing speed. Many pro cyclists can hold between 5 to 5.5 watts per kg for sustained periods (10 to 60 minutes) while climbing.

| | | Power to Weight (Watts per Kg) for a Given Speed (mph) and Grade (%) for a 70 kg (154 lb) Person with a 6.8 kg (15 lb) Bicycle | | | | | | | | | |
|-------------|------|---|------|------|-------|-------|-------|-------|-------|-------|-------|
| Speed (mph) | S | Percent Grade | | | | | | | | | |
| | | 1% | 2% | 3% | 4% | 5% | 6% | 7% | 8% | 9% | 10% |
| 7.5 | 0.39 | 0.63 | 0.87 | 1.11 | 1.35 | 1.60 | 1.84 | 2.08 | 2.32 | 2.56 | 2.80 |
| 10 | 0.64 | 1.00 | 1.36 | 1.72 | 2.08 | 2.44 | 2.80 | 3.16 | 3.52 | 3.88 | 4.24 |
| 12.5 | 0.95 | 1.43 | 1.92 | 2.40 | 2.88 | 3.36 | 3.85 | 4.33 | 4.81 | 5.29 | 5.77 |
| 15 | 1.34 | 1.94 | 2.55 | 3.15 | 3.75 | 4.35 | 4.95 | 5.55 | 6.15 | 6.75 | 7.35 |
| 17.5 | 1.85 | 2.57 | 3.39 | 4.02 | 4.71 | 5.46 | 6.18 | 6.90 | 7.62 | 8.34 | 9.06 |
| 20 | 2.49 | 3.33 | 4.18 | 5.02 | 5.86 | 6.71 | 7.55 | 8.39 | 9.24 | 10.08 | 10.92 |
| 22.5 | 3.25 | 4.21 | 5.17 | 6.13 | 7.09 | 8.05 | 9.01 | 9.97 | 10.93 | 11.89 | 12.85 |
| 25 | 4.12 | 5.23 | 6.42 | 7.61 | 8.79 | 9.99 | 10.76 | 11.85 | 12.94 | 14.02 | 15.11 |
| | 5.24 | 6.62 | 7.85 | 9.04 | 10.25 | 11.46 | 12.66 | 13.87 | 15.08 | 16.29 | 17.50 |

©Allen Lim

Table 4. The calculated VAM or vertical ascent rate in meters per hour at different speeds and % grades. Notice that you're the steeper the

climb the higher the VAM at any given speed. Because, the percentage of power due to gravitational resistance increases at steeper grades, VAM should only be used to calculate power at % grades of 6% or greater. Ideally, an 8 to 10% grade should be the only hills used to estimate power from VAM.

Ascent Rate (Meters per Hour) for a Given Speed (mph) and Grade (%)
for a 70 kg (154 lb) Person with a 6.8 kg (15 lb) Bicycle

| Speed (mph) | Percent Grade | | | | | | | | | |
|-------------|---------------|-----|------|------|------|------|------|------|------|------|
| | 1% | 2% | 3% | 4% | 5% | 6% | 7% | 8% | 9% | 10% |
| 5 | 81 | 161 | 242 | 323 | 404 | 484 | 565 | 646 | 726 | 807 |
| 7.5 | 121 | 242 | 362 | 483 | 604 | 725 | 846 | 966 | 1087 | 1208 |
| 10 | 161 | 323 | 484 | 646 | 807 | 969 | 1130 | 1291 | 1453 | 1614 |
| 12.5 | 201 | 402 | 603 | 804 | 1006 | 1207 | 1408 | 1609 | 1810 | 2011 |
| 15 | 242 | 483 | 725 | 966 | 1208 | 1450 | 1691 | 1933 | 2174 | 2416 |
| 17.5 | 282 | 565 | 847 | 1129 | 1412 | 1694 | 1976 | 2259 | 2541 | 2824 |
| 20 | 321 | 643 | 964 | 1286 | 1607 | 1929 | 2250 | 2571 | 2893 | 3214 |
| 22.5 | 364 | 727 | 1091 | 1455 | 1818 | 2182 | 2545 | 2909 | 3273 | 3636 |
| 25 | 404 | 809 | 1213 | 1618 | 2023 | 2427 | 2831 | 3236 | 3640 | 4045 |

©Allen Lim

Table 5. The percentage of a cyclist's power used to overcome gravitational resistance at different velocities and percent grades. At steeper grades and lower velocities gravitational resistance is a larger contributor to the total power.

Percentage of Total Power Required to Overcome Gravity
for a 70 kg (154 lb) Person with a 6.8 kg (15 lb) Bicycle

| Speed (mph) | Percent Grade | | | | | | | | | |
|-------------|---------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| | 1% | 2% | 3% | 4% | 5% | 6% | 7% | 8% | 9% | 10% |
| 5 | 61.8% | 76.4% | 82.0% | 86.6% | 89.0% | 90.7% | 91.9% | 92.8% | 93.6% | 94.2% |
| 7.5 | 56.6% | 72.3% | 79.7% | 83.9% | 86.7% | 88.7% | 90.1% | 91.3% | 92.3% | 92.9% |
| 10 | 50.6% | 67.2% | 75.5% | 80.4% | 83.7% | 86.0% | 87.8% | 89.1% | 90.2% | 91.1% |
| 12.5 | 44.7% | 61.8% | 70.8% | 76.4% | 80.2% | 82.9% | 85.0% | 86.6% | 87.9% | 89.0% |
| 15 | 39.0% | 56.1% | 65.7% | 71.9% | 76.2% | 79.3% | 81.7% | 83.6% | 85.2% | 86.5% |
| 17.5 | 33.9% | 50.6% | 60.6% | 67.2% | 71.9% | 75.4% | 78.2% | 80.4% | 82.2% | 83.7% |
| 20 | 29.6% | 45.6% | 55.7% | 62.7% | 67.7% | 71.6% | 74.6% | 77.0% | 79.1% | 80.8% |
| 22.5 | 25.6% | 40.7% | 50.8% | 57.9% | 63.2% | 67.3% | 70.6% | 73.3% | 75.6% | 77.3% |
| 25 | 22.3% | 36.5% | 46.7% | 53.5% | 58.9% | 63.3% | 66.8% | 69.7% | 72.1% | 74.2% |

Dr. Allen Lim is an exercise physiologist and coach with Thrive Health Fitness Medicine. He received his doctorate at the

University of Colorado at

Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: Coasting Through

Stage 12: Becoming A Better Climber

By Allen Lim PhD

Thursday July 14th

Route: Briancon to Digne-les-Bains
Type: Mountain Stage
Distance: 187 km or 116 miles
Elevation Change: -588 meters or -1,929 feet loss (Average pt to pt grade = - 0.31%)
Total Feet Climbed: 1,740 meters or 5,709 feet
Average Elevation: 973 \pm 225 meters of 3,194 \pm 737 feet above sea level
Minimum Elevation: 636 meters or 2,087 feet
Maximum Elevation: 1,346 meters or 4,416 feet
Weather 80° to 86°F. Summer is here. It's getting hot.

Climbs:

Cote des Demoiselles-Coiffees (Cat 3): 4.6 km at 4.8% beginning at 54 km point.
 2. Col Saint Jean (Cat 2): 13.2 km at 4% at 75 km point.
 3. Col du Labouret (Cat 4): 2.2 km at 3.2% at 113 km point.
 4. Col du Corobin (Cat 2): 12.4 km at 4.5% at 142 km point.
 5. Col de l'Ome (Cat 4): 2.7 km at 3.9% at 175 km point.

At a Glance:

Stage Placing: 84th
GC Placing: 11th
Time: 04:30:39 (10 min & 33 seconds down on winner David Moncoutie)
Average Speed: 41.5 km/hr or 25.7 mph
Average Power: 231Watts
Total Work: 3,751 Kjoules
Stress to Strain Index: 1.08

Predicted Average Power at For Floyd Alone: 310 Watts

% Savings due to Drafting: 25.5%

Some Thoughts on Previous Thoughts:

On the drive to the finish today, I started thinking a lot more about a question I alluded to in **yesterday's report**. It was the question about how much rider's actually save when they're "drafting" or perhaps better stated, "following wheels" on the climbs. The bottom line is, I think I'm right about the very small 7-watt drafting related saving while climbing on the Galibier yesterday. Though those 7 watts only represented 2% of the total 342 watts required for Floyd to get up the Galibier, the aerodynamic resistance holding him back was only 37 watts or about 11% of the total power. On the other hand, that 7-watt saving represents 19% of the total aerodynamic drag cost. Since aerodynamic resistance increases with the velocity squared, there would be proportionally less savings from drafting at slow speeds. On yesterday's climb the average speed was only 12.7 mph. So the numbers check out. For the most part, trying to draft on yesterday's climb was more of a psychological boost and also a way to help keep one's pace steady. In the end, if the climbing speed is less than 15 mph, there is minimal benefit to drafting. But if it's higher than 15 mph, you better find a wheel and hold on.

I also started thinking a lot about how hard each of these mountain stages are with respect to the climbing load. In particular, it was a comment that Floyd made last night that this stage was going to be one of the easiest climbing days. So I took the ranking I developed based on the distance and steepness of each climb and ranked every single stage with climbing points according to their difficulty. That ranking is presented in table 2. In summary, today's stage only ranked 8th out of 18, with respect to climbing difficulty. More importantly, the hardest and 3rd hardest day of climbing are still to come.

Thoughts on Today's Power:

The most exciting thing to report about today's data is that Floyd hit a new all Tour high for coasting at zero watts of 42 minutes beating stage 7's high by 1 minute. That's right, today Floyd coasted for 42 minutes. Otherwise, today was so average it was kind of weird. In addition, it really looked more like one of the early flat stages than a mountain stage. Check out the numbers in Table 1. If I were just looking at the power data, I wouldn't be able to tell that this was suppose to be a mountain stage. Indeed, of the mountain stage days, the total amount of climbing was the lowest at 5,709 feet. So for today, climbing ability didn't have much to do with the final results.

Floyd's Climbing Program:

Of course, a rider's ability to climb is one of the most if not the most important assets he has at the Tour. For Floyd it was what he focused on the most this year. Consequently, I thought it'd be neat to share exactly what he did this last year to prepare for the hills and outline a little hill climbing training program that others can scale down for their own preparation.

In short, our conversation about training for the mountains early this year went something like this:

Me: So how much do you try to climb each month?

Floyd: About 150,000 vertical feet a month.

Me: You think you can do more?

Floyd: Yeah.

Me: Okay, cool. Then, I think you should do more.

Floyd: Sounds good.

And that was basically it. By the time Floyd got to the Tour, he had averaged 200,000 feet of climbing each month for the three months prior. That's an average of about 8,000 feet of climbing every time he went out to train and more than he had ever accomplished. This might sound like a lot, but if you look at the 9 major mountain stages in this year's Tour and total the elevation gain for each of the categorized climbs, the average is $8,784 \pm 2,710$ feet of climbing with a minimum of 4,882 feet (**today's stage**) and a maximum of 12,953 feet (**stage 15**). That's not counting all the little rollers and uncategorized hills the riders do.

While, it may sound over simplistic, the fact of the matter is, if you want to improve your climbing, you need to climb. The biggest mistake I see riders make in their hill climb training is they don't climb enough. No matter what you do, if you don't spend the time in the mountains you won't improve your climbing.

Just climbing more, however, wasn't the only thing involved in preparing Floyd for the hills. It only served as a baseline for more specific work. Just like it is sometimes important to establish a nice base of volume in the early season before any high intensity, it's sometimes good to just establish a big base of climbing volume. After that volume base is done, it's critical to start forcing yourself to handle a higher sustainable intensity. As you begin to raise that sustainable intensity the next step is attempting to vary the intensity on the climbs. Finally, no matter what you do, you won't climb at your best if you're not at an optimal weight. So if you're a bit or a lot overweight, all things being equal, you'll climb faster if your carrying less weight.

Below is a short review of the steps we took to make Floyd better on the hills as applied to you:

1. Volume

Before you start consulting any rocket scientists about how to improve your hill climbing, first, try climbing more and see what happens. When you do that climbing, don't worry about intensity or how hard you go. Just go as easy as you can to make it to the top and just try getting over the top of as many climbs as possible. If you live in an area that's not too hilly, doing repeats on a single hill is a viable solution. If you live in a place with no hills at all, then you'll just have to work on your overall fitness and not worry about it.

2. Hard and Steady

After you get comfortable just being in the hills a lot more, start focusing on going hard on those hills. Most importantly, on any length of climb, try keeping as high and sustainable pace on that hill as possible. If it's a short hill, then you'll be going harder than a longer hill. In the end, it's important to teach your body to go as hard as possible for any length of climb. Don't worry about blowing up either. Just think of it as positive feedback.

3. Negative Splits

Once you learn how to climb hard and steady, it's time to learn how to bring that intensity up a notch over the top of the climbs. That is, start doing hill intervals where you go a nice moderate tempo on the first half of the climb and then go crazy over the top. It's a great way to teach your body how to redline on the climbs and if you can handle it, it's always better to drop your competitors over the top of a climb so that you can recover from the effort on the descent.

4. Quarter Splits

One of the hardest things to do on a climb is accelerating and decelerating. While many can climb a solid steady pace, forcing someone to surge or being able to deal with someone else's surge can make or break your race. As an example, if you know you can hold 200 Watts on a hill, try breaking that same hill into 4 equal sections. On the first section, ride at 150 Watts, on the second, ride at 250 Watts, on the fourth 150 Watts, and on the last section bring it back up to 250 Watts. Although the average power will be exactly the same, you'll notice that the effort feels considerably harder. Thus, after learning how to negative split (1st half faster than the second half) a hill, it's important to start "quartering" your climbs.

5. Terrain based intervals

In racing, the surges and pace setting is rarely based on some fixed time or distance. Those surges or attacks happen relative to changes in the terrain. Accordingly, after a certain point in training, I usually throw away the clock with respect to intervals on the climbs and have riders simply use terrain features, as their cue for how long and hard different efforts should be. In racing, it's not like riders are going to go all out for a predefined 5 or 10 minutes and then sit up. They'll go hard all the way over the top, or attack on the steepest sections. Accordingly, Floyd will simply imagine how the attacks might play out on a given climb and try to mimic that in training.

6. Pre Climb Throttling

One important aspect of climbing in the Tour is being able to handle the hard place leading into the climb. It's a common tactic for the strong teams to have their non-climbing specialists to give the climbers a "lead out" into the hill. That is, they lift the pace really high coming into the hill to wear people out even more. For Floyd, this meant that by the time we got close to the Tour, I would motor pace him for 5 to 20 minutes before every major climb he did in training.

7. Losing Weight

Though, I'd rather just focus and recommend that people primarily focus on making their engine stronger, you can't ignore the fact that climbing speed is almost entirely dependent on a rider's power to weight ratio. Get down to an optimal weight and you'll go faster uphill. But get too skinny and you may lose valuable muscle mass and power. One final note, if you do decide to try and lose weight, do not try to lose more than a pound a week. In addition, if your friends or family start freaking out about how skinny you are, I would recommend listening to them. In my experience, athletes or anyone driven to succeed, are a poor judges of their own body image and that's all I'll say about that. Happy climbing.

Table 1. Floyd's power data for Stage 12.

Table 2. Stages with mountain points ranked in order of difficulty. The difficulty index is based on the steepness and grade of the climb.

Dr. Allen Lim is an exercise physiologist and coach with Thrive Health Fitness Medicine. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power

| | Stage II | Tour Average | Min | Max |
|-------------------|------------------------------|--------------|-----------------------|--------------|
| Basic Stats | Duration (hrs:min) | 4:31 | 4:30 ± 0:28 | 3:46 (S5) |
| | Distance (miles) | 116 | 121 ± 14 | 107 (S11) |
| | Average Speed (mph) | 25.7 | 27.0 ± 1.79 | 22.3 (S11) |
| | Perception of Effort (1-10) | 5 | 5.5 ± 1.3 | 4 (S2, S3) |
| | Average Cadence (RPM) | 82 | 83.2 ± 3.5 | 81 (S6, S11) |
| Peak Power | Average Power (Watts) | 231 | 241 ± 25 | 206 (S2) |
| | Work from Power (KJoules) | 5751 | 3925 ± 709 | 2855 (S2) |
| | Work from RPE (KJoules) | 4060 | 4456 ± 1316 | 2772 (S2) |
| | Stress to Strain Index | 1.08 | 1.12 ± 0.16 | 0.88 (S3) |
| | 5 sec (Watts) | 922 | 846 ± 84 | 737 (S11) |
| | 30 sec (Watts) | 353 | 357 ± 52 | 463 (S2) |
| | 1 min (Watts) | 487 | 475 ± 48 | 403 (S2) |
| | 5 min (Watts) | 427 | 393 ± 58 | 308 (S2) |
| | 30 min (Watts) | 322 | 307 ± 50 | 249 (S2) |
| | 1 hour (Watts) | 290 | 282 ± 43 | 231 (S2) |
| Zone Distribution | 2 hours (Watts) | 266 | 250 ± 31 | 227 (S2) |
| | Zero Watts (% or min) | 15.4% or 42 | 12.4 ± 2.8% or 33 ± 6 | 26 min (S8) |
| | Recovery (% or min) | 20.3% or 55 | 24 ± 6.4% or 64 ± 16 | 36 min (S11) |
| | Endurance (% or min) | 34.8% or 81 | 33 ± 1.7% or 89 ± 11 | 72 min (S2) |
| | Lactate Threshold (% or min) | 11.7% or 32 | 11 ± 5% or 30 ± 15 | 17 min (S2) |
| | Race Pace (% or min) | 8.0% or 22 | 9 ± 3.3% or 23 ± 10 | 12 min (S2) |
| | Max (% or min) | 4.8% or 13 | 5 ± 1% or 13 ± 4 | 8 min (S2) |
| | Supra-Max (% or min) | 4.8% or 13 | 5.8 ± 1.6% or 16 ± 4 | 12 min (S11) |

©Allen Lim

| RANK | STAGE # | TOTAL SCORE |
|------|---------|-------------|
| 1 | 15 | 397 |
| 2 | 11 | 355 |
| 3 | 14 | 259 |
| 4 | 10 | 258 |
| 5 | 9 | 237 |
| 6 | 16 | 225 |
| 7 | 8 | 160 |
| 8 | 12 | 148 |
| 9 | 19 | 144 |
| 10 | 18 | 130 |
| 11 | 17 | 48 |
| 12 | 6 | 38 |
| 13 | 7 | 26 |
| 14 | 20 | 26 |
| 15 | 3 | 15 |
| 16 | 21 | 8 |
| 17 | 13 | 7 |
| 18 | 5 | 4 |

Today's Stage
Completed Stage

©Allen Lim

Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: Battling Heat And Humidity

Stage 13: Today's weather conditions create challenges

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Friday, July 15th

Route: Miramas to Montpellier

Type: Flat Stage

Distance: 173.5 km or 107.6 miles

Elevation Change: -10 meters or -55 feet loss (Average pt to pt grade = - 0.01%)

Total Feet Climbed: 351 meters or 1,152 feet

Average Elevation: 79.1 ± 49 meters of 260 ± 160 feet above sea level

Minimum Elevation: 10 meters or 33 feet

Maximum Elevation: 185 meters or 607 feet

Weather: 80°F and 78% Humidity. Right on the high risk exercise edge.

Climbs:

1. Col de La Vavede (Cat 4): 1.1 km at 6.1% beginning at 25.4 km point.

At a Glance:

Stage Placing: 78th

GC Placing: 11th

Time: 03:43:14 (Same time as winner Robbie McEwan)

Average Speed: 46.6 km/hr or 28.9 mph

Average Power: 213Watts

Total Work: 2,853 Kjoules

Stress to Strain Index: 1.41

Predicted Average Power at For Floyd Alone: 424 Watts

% Savings due to Drafting: 50.2%

Not That Easy:

Today's power data is really interesting as Floyd hit a new low for time (3:43:14) and for work (2,853 Kjoules), but had a very high stress to strain index. There are a number of reasons why he thought the stage felt a lot harder than it actually looks from the power data. Those reasons include fatigue, a huge number of very short (5 to 10 second) accelerations, and most notably today's killer combination of clear skies, heat, and humidity.

After the first 3 minutes, the speed came up and stayed up for the entire race. With close to two weeks of racing (the last 3 in the Alps) it was amazing for me to see how high the speed was. At 28.9 mph today's speed was exactly the same speed as the average of the first 6 flat stages. But for most of the riders, it felt a lot harder and a lot faster.

Unlike those first flat stages the clouds and rain that followed the riders for the first week made way for an oppressively bright sun and a heat and humidity combination that is considered to be "high risk" conditions for exercise (Table 2).

The Bottle & Weight Count:

Besides racing today, all of the riders pounded through a whole lot of water bottles filled with the team's typical ice-cold carbohydrate and electrolyte solution. Today Floyd thinks that he drank about 12 bottles while on the bike. He thinks, because when you're going that fast in conditions like today, you're not really counting, just doing.

With each bottle holding 500 ml's of fluid, that's 6 liters or just over 1.5 gallons of fluid consumed while on the bike today. Still, when he got off the bike at the end of the stage he was 3 kilograms lighter than he was after breakfast this morning. That means he lost 3 liters (1 kilogram of water = 1 liter of water) of fluid by the end of today's race or close to 4.5% of his body weight despite all those bottles he went through on the bike. If we add it all up, then Floyd's total sweat loss was equal to 9 liters or 2.4 liters per hour.

The last two figures I just presented are extremely important to keeping Floyd properly hydrated. For most individuals, a fluid loss that is equal to 5 to 10% of their body weight is enough to cause signs of heat exhaustion. Although, Floyd has felt fine after long days of training with up to a 7% loss in body weight, to be conservative we make it a goal that while racing he tries not to lose more than 5% of his body weight. Today he just barely made that cut off.

Unlike those first flat stages the clouds and rain that followed the riders for the first week made way for an oppressively bright sun and a heat and humidity combination that is considered to be "high risk" conditions for exercise (Table 2).

The Bottle & Weight Count:

Besides racing today, all of the riders pounded through a whole lot of water bottles filled with the team's typical ice-cold carbohydrate and electrolyte solution. Today Floyd thinks that he drank about 12 bottles while on the bike. He thinks, because when you're going that fast in conditions like today, you're not really counting, just doing.

With each bottle holding 500 ml's of fluid, that's 6 liters or just over 1.5 gallons of fluid consumed while on the bike today. Still, when he got off the bike at the end of the stage he was 3 kilograms lighter than he was after breakfast this morning. That means he lost 3 liters (1 kilogram of water = 1 liter of water) of fluid by the end of today's race or close to 4.5% of his body weight despite all those bottles he went through on the bike. If we add it all up, then Floyd's total sweat loss was equal to 9 liters or 2.4 liters per hour.

GDC00820

The last two figures I just presented are extremely important to keeping Floyd properly hydrated. For most individuals, a fluid loss that is equal to 5 to 10% of their body weight is enough to cause signs of heat exhaustion. Although, Floyd has felt fine after long days of training with up to a 7% loss in body weight, to be conservative we make it a goal that while racing he tries not to lose more than 5% of his body weight. Today he just barely made that cut off.

3) Pre-hydrate as much as you can before exercise. One strategy I recommend to riders is drinking an entire water bottle while they are waiting at the line. The extra fluid in their gut serves as a reservoir that helps to delay dehydration. In addition, that extra fluid also creates a bit of a pressure gradient that helps subsequent fluid enter the body faster.

4) Acclimatize to the heat before competing in the heat. This means that you need to pre-expose yourself to heat if you want to be able to handle exercise in the heat. The best way to adapt to the heat is to do short and very intense workouts in the heat that elicit a very high sweat rate. Intensity is a stronger stimulus for adapting to the heat than volume.

5) Stay out of the heat. If the combination of temperature and humidity are in the risky zone, then it's sometimes best to just avoid exercise until it's cooler. In addition, if you have to compete in the heat, it's always best to stay out of the heat for as long as possible before your competition. This will help prevent unnecessary fatigue and actually help your performance.

6) Pre-cool before exercise. Getting cool before exercising in the heat can actually help your performance in the heat for short events (10 to 30 minutes). While I won't say much about it now, stay on the look out for some unique technology Floyd will be unveiling for the final time trial.

Table 1. Floyd's power data for stage 13.

| | Stage 13 | Tour Average | Min | Max |
|------------------------------|--------------|------------------------|------------------|--------------|
| Basic Stats | | | | |
| Duration (hrs:min) | 3:43 | 4:26 ± 0:30 | 3:43 (S13) | 5:04 (S8) |
| Distance (miles) | 107.6 | 120 ± 14 | 107 (S11) | 144 (S8) |
| Average Speed (mph) | 29.9 | 27.0 ± 2.79 | 22.3 (S11) | 30.1 (S5) |
| Perception of Effort (1-10) | 6 | 5.5 ± 1.2 | 4 (S2, S5) | 8 (S10) |
| Average Cadence (RPM) | 53 | 55 ± 3.4 | 31 (S6, S11) | 90 (S2) |
| Average Power (Watts) | 213 | 238 ± 25 | 200 (S2) | 283 (S11) |
| Work from Power (KJoules) | 2553 | 3925 ± 709 | 2853 (S13) | 4910 (S11) |
| Work from RPE (KJoules) | 4018 | 4416 ± 1283 | 2772 (S2) | 6067 (S11) |
| Stress to Strain Index | 1.41 | 1.15 ± 0.18 | 0.88 (S3) | 1.48 (S10) |
| Peak Power | | | | |
| 5 sec (Watts) | 594 | 550 ± 50 | 757 (S11) | 965 (S8) |
| 30 sec (Watts) | 594 | 561 ± 50 | 465 (S2) | 645 (S9) |
| 1 min (Watts) | 509 | 476 ± 47 | 303 (S2) | 553 (S9) |
| 5 min (Watts) | 351 | 389 ± 56 | 308 (S2) | 478 (S9) |
| 30 min (Watts) | 258 | 302 ± 49 | 249 (S2) | 386 (S8) |
| 1 hour (Watts) | 242 | 278 ± 43 | 231 (S2) | 359 (S11) |
| 2 hours (Watts) | 253 | 256 ± 30 | 227 (S2) | 315 (S11) |
| Zone Distribution | | | | |
| Zero Watts (% or min) | 18.0 % or 40 | 13 ± 3% or 33.5 ± 6 | 26 min (S3) | 42 min (S12) |
| Recovery (% or min) | 26.0% or 58 | 24 ± 8% or 63 ± 15.6 | 36 min (S11) | 39 min (S7) |
| Endurance (% or min) | 30.9% or 69 | 33 ± 1.7% or 86.6 ± 12 | 72 min (S2) | 102 min (S8) |
| Lactate Threshold (% or min) | 7.7% or 17 | 11 ± 5% or 29 ± 14.6 | 17 min (S2, S13) | 66 min (S11) |
| Race Pace (% or min) | 6.4% or 14 | 8.3 ± 3.1% or 22 ± 10 | 12 min (S2) | 44 min (S11) |
| Max (% or min) | 4.7% or 10 | 5 ± 1% or 13 ± 4 | 8 min (S2) | 22 min (S8) |
| Supra-Max (% or min) | 6.3% or 14 | 5.8 ± 1.0% or 15 ± 5.4 | 12 min (S11) | 24 min (S8) |

©Allen Lim

Table 2. Temperature and humidity combinations considered dangerous to exercise in.

High Risk Exercise Combinations

| Temp °F | %Humidity |
|---------|-----------|
| 70 | 100 |
| 75 | 90 |
| 80 | 80 |
| 85 | 70 |
| 90 | 60 |
| 95 | 50 |
| 100 | 40 |
| 105 | 30 |

Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

©Allen Lim

Bicycling

Powerfeed: The Hardest Day Yet

Stage 14: Now, it's not necessarily about who can produce the most power, but who can hold on to the most power over the rest of the race

By Allen Lim PhD

Route: Adge to Ax-3 Domains
Type: Mountain Stage
Distance: 220.5 km or 107.6 miles
Elevation Change: 1,359 meters or 4,459 feet gain (Average pt to pt grade = 0.61%)
Total Feet Climbed: 3,209 meters or 10,529 feet
Average Elevation: 440 ? 474 meters or 1,444 ? 1,554 feet above sea level
Minimum Elevation: 13 meters or 43 feet
Maximum Elevation: 2001 meters or 6,565 feet
Weather: 85°F to 92°F and 35 to 70% Humidity. Another hot and dangerous day.

Climbs:

1. Col de Villerouge (Cat 4): 6.3 km at 3.6% beginning at 84 km point.
2. Col de Bedos (Cat 4): 3.3 km at 4.4% beginning at 95 km point.
3. Col des Fourches (Cat 4): 2.2 km at 4.5% beginning at 102 km point.
4. Col du Paradis (Cat 3): 5.8 km at 4.1% beginning at 109 km point.
5. Port de Pailheres (HD): 15.1 km at 8.1% beginning at 176 km point.
6. Ax-3 Domains (Cat 1): 7.9 km at 8.3% beginning at 212 km point.

At a Glance:

Stage Placing: 6th
GC Placing: 6th
Time: 05:45:14 (1 min & 31 seconds down on winner Georg Totschnig)
Average Speed: 38.3 km/hr or 23.8 mph
Average Power: 262 Watts
Total Work: 5,427 Kjoules
Stress to Strain Index: 1.72

Big Day:

By all accounts today was the hardest day of racing in the Tour. So far this was the longest day on the bike (5:45 hr:min), the most work (5,427 Kjoules), the most time at race pace intensity (50 min), and the most time at an endurance intensity (115 min) range for Floyd. Most importantly, Floyd gave the overall race his highest perceived exertion rating of 8.5 and 10's for the final two climbs up Pailheres and Ax-3 Domains. Everyone was at their limit on the last climb and as Floyd put it, "racing just doesn't get harder than that." With an even harder stage 15 to come, I don't think there will be that much room for negotiating GC spots after the riders finish tomorrow. As hard as today was, it is worth noting that the power outputs on the final climbs in today's stage were not greater than what Floyd has accomplished in training in the recent past. The difference is that we are now two weeks into racing. Add the heat, stress, and the fact that everyone is getting tired and you have a situation where results are not necessarily about who can produce the most power, but who can hold on to the most power over the course of the race.

At this point, it would be difficult to imagine that we will see any new highs for peak power output. In fact, outside of the increased work and time spent in the two intensity zones mentioned earlier, today's data, while greater than average, is by no means the best of the Tour despite today being the hardest.

Climbing and Cadence:

There are two ways to produce more power on a bicycle. You can either push harder or pedal faster (i.e., increase torque or increase your rpm). The age-old question has been, which of the two is easier or more efficient. The short answer is that if you want to produce more power, you need to do both. That's because, as we increase our power output, how hard we can push tends to plateau, so very high power outputs tend to be more efficient or associated with higher cadences. At the same time, the primary difference between a professional cyclist and a recreational cyclist is that the professional cyclist can simply push harder. They don't necessarily just pedal faster all the time. In fact, for most cyclists, regardless of their fitness, there is almost no detectable change in efficiency at a cadence between 80 to 100 rpm. Although much is often made between the cadence difference of Lance Armstrong and Jan Ulrich, they are simply on either end of that neutral cadence range and when riding side by side up a steep climb they're both producing nearly equal and extraordinarily high power outputs for their body weight.

Thus, the real key to climbing and cadence isn't necessarily pedaling at a particular cadence. It's about having the gears so that regardless of terrain, you can keep your cadence in a neutral range of 80 to 100 rpm. It's also about finding out what works best for you as an individual. Experimenting with a power meter while riding up steep grades is the most objective way to figure that out. At a constant power output simply try changing your gears so that you ride that power output at different cadences. You'll be surprised that you can actually feel that there is a difference in how hard it feels to hold that same exact power output at different cadences. Practically speaking, whichever cadence feels the best for you is probably the one you should go with.

In general, I find that most riders are simply over geared for the hills. The reality is, most bikes sold today are geared for professional cyclists not your average Joe or competitive amateur. After all, the fact that a pro's bike here in the Tour has the same gearing as that sold in your local bike shop should raise some questions. I mean, ego aside, you wouldn't use the same transmission on an engine that is only half as powerful. So for most cyclists, instead of the standard tooth crank combination of 53 x 39, I recommend a "compact crank" combination of 50 x 34. For the everyday cyclist, it just works better because it helps keep you in that normal cadence range on steep climbs. But if you're not ready to let go of those big chain rings, then it might interest you to know that in tomorrow's stage - the hilliest of all the Tour stages - Floyd and his teammates will be going "compact." In the end, it's not about pride it's about speed.

Table 1. Floyd's power data for stage 14.



| | | Stage 14 | Tour Average | Min | Max |
|-------------------|------------------------------|--------------|-------------------------|------------------|---------------|
| Basic Stats | Duration (hrs:min) | 5:45 | 4:32 ± 0:37 | 3:43 (S13) | 5:45 (S14) |
| | Distance (miles) | 137 | 121 ± 14 | 107 (S11) | 144 (S8) |
| | Average Speed (mph) | 23.8 | 26.9 ± 2.7% | 22.3 (S11) | 30.1 (S5) |
| | Perception of Effort (1-10) | 8.5 | 5.5 ± 1.2 | 4 (S2, S3) | 9 (S10) |
| | Average Cadence (RPM) | 81 | 85 ± 3.3 | 81 (S6, S11) | 90 (S2) |
| | Average Power (Watts) | 262 | 240 ± 25 | 206 (S2) | 285 (S11) |
| Peak Power | Work from Power (KJoules) | 5427 | 3960 ± 845 | 2853 (S13) | 5427 (S14) |
| | Work from RPE (KJoules) | 9321 | 4825 ± 1871 | 2772 (S2) | 9321 (S14) |
| | Stress to Strain Index | 1.72 | 1.19 ± 0.24 | 0.88 (S3) | 1.72 (S14) |
| | 5 sec (Watts) | 904 | 855 ± 79 | 757 (S11) | 965 (S8) |
| | 30 sec (Watts) | 578 | 562 ± 48 | 465 (S2) | 645 (S9) |
| | 1 min (Watts) | 489 | 478 ± 44 | 403 (S2) | 555 (S9) |
| | 5 min (Watts) | 445 | 394 ± 56 | 308 (S2) | 478 (S9) |
| | 30 min (Watts) | 379 | 309 ± 52 | 249 (S2) | 386 (S3) |
| | 1 hour (Watts) | 340 | 284 ± 44 | 231 (S2) | 359 (S11) |
| | 2 hours (Watts) | 310 | 261 ± 33 | 227 (S2) | 315 (S11) |
| Zone Distribution | Zero Watts (% or min) | 9.6 % or 33 | 12.7 ± 3% or 33.5 ± 5.7 | 36 min (S8) | 42 min (S12) |
| | Recovery (% or min) | 17.1% or 59 | 23.7 ± 6% or 63 ± 14.8 | 36 min (S11) | 36 min (S7) |
| | Endurance (% or min) | 33.4% or 113 | 33 ± 1.7% or 86.6 ± 12 | 72 min (S2) | 115 min (S14) |
| | Lactate Threshold (% or min) | 16.7% or 55 | 11.3 ± 4.8% or 31 ± 16 | 17 min (S2, S13) | 66 min (S11) |
| | Race Pace (% or min) | 14.6% or 50 | 8.8 ± 3.6% or 25 ± 13 | 12 min (S2) | 50 min (S14) |
| | Max (% or min) | 4.6% or 16 | 4.9 ± 1.1% or 13 ± 4 | 8 min (S2) | 22 min (S8) |
| | Supra-Max (% or min) | 4.0% or 14 | 5.6 ± 1.0% or 15 ± 3.2 | 12 min (S11) | 24 min (S8) |

©Allen Lim

Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: Fighting Fatigue

Stage 15: Finding your limit is the best thing possible

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Sunday, July 17th

Route: Lezat-sur-Leze to Saint-Lary-Soulan

Type: Hardest Mountain Stage on Paper

Distance: 205.5 km or 127.4 miles

Elevation Change: 1,475 meters or 4,839 feet gain (Average pt to pt grade = 0.72%)

Total Feet Climbed: 4,869 meters or 15,975 feet

Average Elevation: 783 ± 428 meters of 2,570 ± 1,403 feet above sea level

Minimum Elevation: 205 meters or 673 feet

Maximum Elevation: 1,680 meters or 5,512 feet

Weather: 86°F and 55% Humidity. The heat is still oppressive and the sun garish. But not as bad as yesterday.

Climbs:

1. Col de Portet-d'Aspet (Cat 2): 5.9 km at 6.9% beginning at 89.1 km point.
2. Col de Mente (Cat 1): 7 km at 8.1% beginning at 93.5 km point.
3. Col du Portillon (Cat 1): 8.4 km at 7.3% beginning at 129.1 km point.
4. Col de Peyresourde (Cat 1): 13 km at 7% beginning at 149 km point.
5. Col de Val-Louron-Azet (1): 7.4 km at 8.3% beginning at 182.1 km point.
6. St. Lary Soulan (HC): 10.3 km at 8.3% beginning at 195.2 km point.

At a Glance:

Stage Placing: 19th

GC Placing: 7th

Time: 06:16:12 (9 min & 34 seconds down on winner George Hincapie)

Average Speed: 32.8 km/hr or 20.3 mph

Average Power: 249 Watts

Total Work: 5,620 Kjoules

Stress to Strain Index: 2.01

Looking Ahead:

We all knew that today was going to be a difficult and decisive day. I haven't had a chance to catch up with Floyd in depth yet, but I know he gave it his all. Unfortunately, he had a mechanical problem on the final climb and had to switch bikes. It cost him some time, but that's bike racing. If anything, it looks like Floyd will finish this race in the top ten and while I know he expected more from himself, every day of this Tour has been an invaluable learning experience and I couldn't be more proud of what he's accomplished so far.

With the power data we've already collected, we've gained some clear-cut knowledge about ways we can improve his preparation and race for next year. But in the short term, it's time to really start thinking about the final **individual time trial**, to get some well-deserved rest tomorrow, and to look for every opportunity to move forward over this last week. Today may have been the hardest day in the Tour, but the race is far from over and this last week is going to be a difficult one.

The Numbers:

In short, today we saw the most time (6 hrs & 16 min), most work (5,620 Kjoules), highest rating of perceived exertion (10 out of 10), most work based on perceived exertion (11,286 Kjoules), highest stress to strain index (2.01), most time at lactate threshold (69 min), the slowest speed (20.3 mph), and surprisingly, the most time at zero watts (54 min).

In addition, we did not see any new highs for peak power output. Though Floyd's peak power values were, for the most part, all above average, they were not nearly as close to the high power outputs we saw in the earlier mountain stages. As Floyd explains it, at this stage of the game, all of the riders have pretty much unloaded their peak efforts. Now there's just one sustainable power range to hang on to.

While this isn't exactly true, it is interesting to see that for the major mountain stages, the time spent in different intensity zones was incredibly consistent. In some ways it does appear that for any given zone, there is a fixed amount of time Floyd can spend in each before cashing out. Of particular interest to me is how similar the time spent at the top end anaerobic or "supra-maximal" intensity zones has been. Interesting, because it's the ability to go in the "red" and follow hard attacks that seems to distinguish Armstrong from GC rivals like Ulrich, Leipheimer, Basso, and Floyd. Anecdotal and theoretically, it's been said that only the truly aerobic based riders can win the Tour as they are the ones who, while not having the highest peak power outputs, are the ones who fatigue the least and who are able to sustain consistently high power outputs over many hours and many days.

In the Armstrong era of racing, however, it is clear that the combination of an incredible aerobic capacity and an extraordinarily unique ability to accelerate into very high "red zone" or supra-maximal intensity ranges has been a fundamental aspect of Armstrong's victories. While every racer intuitively understands that if they spend too much time in these very high intensity ranges that their day could be over before it even starts, it's unknown how much training can push this fixed limit back, if improving anaerobic capacity inhibits one's aerobic capacity (or vice versa), or if all of these attributes are simply genetically fixed. Whatever the answer, the very small variability in the supra-maximal data we've collected so far does highlight that some sort of limit has been consistently reached in this year's Tour. For us, finding limits is the best thing possible because you can never push back what you can't see.

Causes of Fatigue:

While I'm on the topic of rate limiters and since I've already talked a bit about how fatigue at this stage of the Tour may have put a cap on peak power outputs, I thought

GDC00824

I'd talk a little bit about what causes fatigue. In effect, what are the different mechanisms or reasons for why we get tired when we exercise?

1. Accumulation of Metabolic Product:

If we're talking about high intensity exercise, or all out exercise lasting for seconds to minutes, the primary reason for fatigue is the accumulation of metabolic products - namely lactic acid.

Our bodies have two basic ways of making energy - aerobically (oxidative) by burning fat, carbohydrate, and sometimes protein with oxygen or anaerobically (non-oxidative) by breaking down stored carbohydrate in muscle without oxygen.

Whether we produce energy using oxidative or non-oxidative energy pathways is not necessarily dependent upon whether oxygen is or isn't present. Rather, it's dependent on our power output or how quickly we need energy (i.e., the relative exercise intensity). This is because non-oxidative energy pathways have significantly fewer steps and thus can produce a limited amount of energy extremely fast. Think of it as the fast lane in Germany (This analogy wouldn't work in the U.S.). You can only stay in that lane for very short periods of time before moving over but while you're in that lane you're hauling.

Unfortunately, hauling in the fast lane has a negative consequence in the form of lactic acid accumulation. That is, whenever you produce energy anaerobically (and you're almost always producing at least some of your energy anaerobically when exercising), your body produces lactic acid as the by-product. In most cases, your body is always producing and removing lactic acid, but when you're redlining your body can't remove that lactic acid as fast as it's made. As a result, the acid associated with the lactate (lactate in and of itself does not cause fatigue) can quickly build up and actually inhibit muscular contractions as well as cause the sensation of pain (i.e., "The Burn") in working muscle. What is interesting and what I alluded to earlier is that there seems to be a fixed number of times you can "pass" in the fast lane before that entire lane just shuts down and you're forced to retreat to that slow moving but ever consistent "long haul trucker speed." I don't know the answer to this but the answers could be related to how the accumulation of lactic acid or other metabolic waste products effect some other variables listed below.

2. Depletion of Substrate:

The flip side of accumulating a metabolic by-product that causes fatigue is running out of metabolic substrates or fuel. In short, if the fumes don't get to you first, you'll probably run out of gas later. The type of gas that is the most critical rate limiter is carbohydrate, especially the carbohydrate stored in working muscle in a form we call glycogen.

Though endurance athletes use fat as a primary fuel source and protein can also help to contribute to energy metabolism, fat and protein are essentially unlimited, while glycogen or the carbohydrate you can ingest through your gut is fixed. More importantly, once carbohydrate is depleted, athletes fatigue regardless of how much fat or protein they have in reserve. This is because in normal conditions your central nervous system is fueled entirely by carbohydrate or your blood sugar. So if you've ever "bonked" or "hit the wall" during exercise, it's because you've run out of carbohydrate, your blood sugar has dropped, and your brain and central nervous system are depleted.

Another reason why running out of carbohydrate is so devastating is because the breakdown of carbohydrate is also important to fat metabolism. Thus, when you're out of carbohydrate, your ability to use fat as a fuel is impaired despite the fact that even the scrawny Tour cyclist has ample fat stores to keep him going. The following analogy isn't exactly biochemically correct, but think of it this way. Your metabolism is like a burning candle where carbohydrate is the wick and fat is the wax. If you just burn the wick, it burns hot and it burns fast. If you just burn the fat, all you get is a big waxy mess. But put the two together and you've got a steady flame. Thus, it is sometimes said that, "fat burns in a carbohydrate flame."

For most pros in the Tour, the best way to maintain carbohydrate stores is to eat a high percentage of carbohydrate all the time and to eat a lot of carbohydrate immediately after exercise. While racing, ingesting as much carbohydrate in the form of energy drinks, coke, pastries, gels, candy bars, energy bars, and fruit is critical to keeping the machinery going as the intensities and energy expenditures are so high that there would be no way to ever store enough carbohydrate or glycogen to get any of these cyclists through a single day on the bike here, let alone 21 days. With this in mind, we often say that the most critical asset a Tour de France cyclist has is an iron gut. In fact, it's probably not a stretch to say that for many of these athletes, their gut's (i.e., the small intestine) ability to process and transport valuable carbohydrate and fluid into the body is probably the most critical determinant of fatigue. One final substrate that may possibly limit these cyclists is stored creatine phosphate - a compound important for producing energy anaerobically. Though there is currently no evidence that creatine phosphate loading improves aerobic performance, there is evidence that it can help with maximal strength performance. The unknown question is whether or not more creatine would help with repeated top end accelerations and attacks. My instinct is that it would not, as there are some other potentially negative side effects for endurance athletes loading on too much creatine. But who knows. Maybe it's this substrate or lack of it that shuts down the fast lane.

3. Neural:

When we think about sports, we think of things like our muscles, heart, and lungs as rate limiting our performance. There is good evidence, however, that fatigue in both strength athletes and endurance athletes is not related to those "peripheral" parts, but to the electrical wiring or nervous system that controls it all.

The classic example of this is an athlete wired up with external electrodes who, after reaching a point where his muscles are completely fatigued, is able to do more work when those same tired muscles are externally stimulated with an electrical jolt that mimics our own nervous system. Thus, it may not be our muscles that get fatigued but our nerves. This could be related to something as simple as the balance of sodium and potassium in our bodies that are critical to creating the electrical signal that run down our nerves. Or it could be valuable neurotransmitters that get depleted or lost after a long day of hopping junctions from one nerve cell to another. In fact, there is some evidence that serotonin re-uptake inhibitors - drugs normally taken for depression - can actually help athletic performance by limiting neural fatigue. Finally, I have to mention that the agonizing and completely fatiguing exercise induced muscle cramp may indeed be a neural issue. Of course, this could be directly related to how electrolytes like sodium and potassium control nerve impulses. However, it's probably more related to that simple "knee jerk" reflex that occurs when a doctor taps your knee with that little hammer. Inside each muscle is a very small set of fibers called intrafusal fibers that tell your nervous system whether your muscle is shortening or lengthening. It's this delicate fiber that gets stretched when your knee is hit with the doctors hammer and which sends a reflex signal through your spine that makes your quad contract. It's theorized that these little intrafusal fibers fatigue before the larger muscle fatigues, thereby causing an electrical storm that causes an uncontrolled muscle cramp.

4. Endocrine:

Over the course of many days of hard training or racing like the athletes are facing here, it is common if not inevitable that our endocrine system actually fatigues. That is, the hormonal pathways responsible for producing vital hormones such as testosterone actually get tired and stop producing normal amounts of hormones important for recovery, immune function, and normal health. As an example, this endocrine fatigue is thought to be a major player in the bone loss and loss of menstrual cycle in many elite female endurance runners. For some professional cyclists we know that bone loss is a real and significant health issue. And while they don't lose their menstrual cycle, they do lose their libido. Just ask their wives.

5. Heat Load:

In the last few days, this had to be a major factor causing fatigue in many of the cyclists. In fact, over heating and becoming dehydrated is a factor in performance that just can't be ignored. So have a drink and stay cool cause there's still one more week of hard racing left to go.

Table 1. Floyd's power data from stage 15.

*Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power*

| | Stage 15 | Hour Average | Min | Max |
|-------------------|------------------------------|--------------|--------------------------|------------------|
| Basic Stats | Duration (hrs:min) | 0:16 | 4:30 ± 0:46 | 3:43 (S13) |
| | Distance (miles) | 127.4 | 121 ± 13 | 107 (S11) |
| | Average Speed (mph) | 20.3 | 26.3 ± 3.25 | 20.3 (S15) |
| | Perception of Effort (1-10) | 10 | 6.1 ± 1.9 | 4 (S2, S3) |
| | Average Cadence (RPM) | 84 | 85 ± 3.1 | 81 (S6, S11) |
| | Average Power (Watts) | 249 | 241 ± 24 | 206 (S2) |
| | Work from Power (KJoules) | 5620 | 4088 ± 933 | 2853 (S13) |
| | Work from RPE (KJoules) | 11,386 | 5322 ± 2534 | 2772 (S2) |
| Peak Power | Stress to Strain Index | 2.01 | 1.26 ± 0.32 | 0.88 (S3) |
| | 5 sec (Watts) | 792 | 850 ± 76 | 757 (S11) |
| | 30 sec (Watts) | 540 | 560 ± 46 | 465 (S2) |
| | 1 min (Watts) | 496 | 479 ± 41 | 403 (S2) |
| | 5 min (Watts) | 435 | 397 ± 55 | 308 (S2) |
| | 30 min (Watts) | 361 | 313 ± 52 | 249 (S2) |
| | 1 hour (Watts) | 332 | 288 ± 45 | 231 (S2) |
| | 2 hours (Watts) | 300 | 264 ± 33 | 227 (S2) |
| Zone Distribution | Zero Watts (% or min) | 14.3 % or 54 | 12.8 ± 3% or 35.2 ± 8.0 | 26 min (S8) |
| | Recovery (% or min) | 17.2% or 65 | 23.2 ± 6.2% or 63 ± 14.2 | 36 min (S11) |
| | Endurance (% or min) | 29.7% or 112 | 32.6 ± 1.8% or 91.1 ± 15 | 72 min (S2) |
| | Lactate Threshold (% or min) | 18.5% or 69 | 11.9 ± 5.0% or 34.6 ± 19 | 17 min (S2, S13) |
| | Race Pace (% or min) | 11.8% or 44 | 9.1 ± 3.5% or 36 ± 13 | 12 min (S2) |
| | Max (% or min) | 4.6% or 17 | 4.9 ± 1.1% or 13.7 ± 3.9 | 8 min (S2) |
| | Supra-Max (% or min) | 4.1% or 15 | 5.5 ± 1.1% or 15 ± 3.1 | 13 min (S11) |

©Allen Lim

meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: Lost Computer

Stage 16: The PowerTap took a flyer, leaving Floyd riding commando

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Mourenx to Pau
Type: Mountains with Flat Finish
Distance: 180.5 km or 112 miles
Elevation Change: 98 meters or 322 feet gain (Average pt to pt grade = 0.05%)
Total Feet Climbed: 2,392 meters or 7,848 feet
Average Elevation: 783 ± 428 meters or 2,570 ± 1,403 feet above sea level
Minimum Elevation: 112 meters or 367.5 feet
Maximum Elevation: 1,709 meters or 5,607 feet
Weather: 78°F and 69% Humidity

Climbs:

1. Col d'Ichère (Cat 3): 4.4 km at 6.2% beginning at 46.1 km point.
2. Col de Marie-Blanche (Cat 1): 9.3 km at 7% beginning at 61.2 km point.
3. Col d'Aubisque (Cat HC): 16.5 km at 7.2% beginning at 92 km point.
4. Cote de Pardies-Pietat (Cat 4): 2.6 km at 5.2% beginning at 158.4 km point.

At a Glance:

Stage Placing: 44th
GC Placing: 8th
Time: 04:42:04 (3 min & 24 seconds down on teammate Oscar Pereiro)
Average Speed: 38.6 km/hr or 23.9 mph
Average Power: Floyd thinks maybe 230 to 240 Watts
Total Work: 3,976 Kjoules based on Floyd's guess
Stress to Strain Index: 1.60
Today's Race:

After Sunday Oscar Pereiro was fired up to go out on the attack again today. He was so fired up to make something happen and rode what I think is one of the most incredible races ever. The team was in an emotional frenzy when he crossed the line. It was pretty cool.

Today's Power Data:

The computer fell off the bike. It's embarrassing to say, but that's what happened. About 10 km from the line through a really bumpy section of road, the computer came loose from the bracket and the next thing Floyd knew he was riding commando style. We thought about going back to get it, but as the saying goes, "when your keys fall in molten lava, you just need to let them go...just let them go."



And so, I guess for today, that's exactly what I have to do. It's a serious bummer for both Floyd and myself, because the climbs on today's stage were hard. He says that on the last 3 km of the Col de Marie-Blanche there were consistent 12% pitches. He also thinks there could've been some new peaks for the 5 to 10 minute range, but beyond that everything, at least for the climbing, was really similar to the previous stages. So we're going to go back later and look at video of the climbs and based on the ascent rates, back calculate the average power for the climbs. How we are going to do this is actually pretty interesting.

Yesterday I got to meet the owner of Mat Sport Timing. He operates the photo finish system here at the Tour and came to us because he's been seeing Floyd cross the line everyday with the PowerTap and was really stoked that he was doing the entire Tour with it (He gave me some pictures of Floyd crossing the line in this article). Anyway, he's hooking us up with a system that searches the time code on the video here at the Tour. It will allow us to sync the clock on the PowerTap to the exact time in video. Not only will we be able to search today's data, and based on the course map make some invaluable assessments of the day, but we'll also be able to go back and see what was going on for any given piece of power data. I guess that makes me feel a little bit better about the PowerTap computer getting launched today.

©Allen Lim

Other Thoughts:

I was going to search Floyd's power data today with a specific bent on examining whether there are any signs of over-reaching and over-training in his data from the last few months. Since the data took a flyer, my brain sort of followed, so I'll save any deep thoughts on that topic for tomorrow. Until then, I'll leave you all with a controversial teaser. There is no such thing as overtraining...just under-resting.

Bicycling

Powerfeed: A Stage Filled With Contrast

Stage 17: The longest stage of the Tour produces one of the lowest power outputs

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Pau to Revel
Type: Flat with some rollers
Distance: 239.5 km or 148.5 miles
Elevation Change: -2 meters or -7 feet loss (Average pt to pt grade = -0.001%)
Total Feet Climbed: 1,042 meters or 3,419 feet
Average Elevation: 289 ? 61 meters of 948 ? 201 feet above sea level
Minimum Elevation: 185 meters or 607 feet
Maximum Elevation: 407 meters or 1,335 feet
Weather: 84°F and 48% Humidity

Climbs:

1. Cote de Baleix (Cat 3): 1.6 km at 7.1% beginning at 19.9 km point.
2. Cote de Betbeze (Cat 4): 2.2 km at 6.4% beginning at 86.3 km point.
3. Col d'Aubisque (Cat 4): 1.8 km at 4.9% beginning at 157.7 km point.
4. Cote de Pardies-Pietat (Cat 3): 2.7 km at 5.1% beginning at 229.7 km point.

At a Glance:

Stage Placing: 33rd
GC Placing: 9th
Time: 06:16:12 (22 min & 48 seconds down on winner Paolo Savoldelli)
Average Speed: 39.5 km/hr or 24.5 mph
Average Power: 198 Watts
Total Work: 4,326 Kjoules
Stress to Strain Index: 1.26

Wind Up:

This was a strange race day. I guess you could say it was filled with a lot of contrast. At only 198 watts, it was Floyd's lowest average power output for the entire tour. It was, however, the longest race of the Tour at 148.5 miles and one of the longest at just over 6 hours for Floyd.

Initially, I thought that the low average power output was due to a combination of the duration and the relatively flat roads. Generally speaking, the longer the race the lower the power output. And as we saw from the flat stages in the **first week**, the tremendous savings from drafting can really drop the average power output. To see if this was the case, I broke down the race by each hour and found something pretty interesting. In the first hour, Floyd's average power output was 259 watts, the second hour was 168 watts, the third hour was 170 watts, the fourth hour was a measly 141 watts, the fifth hour was 175 watts, and the last hour was 272 watts. Over the last hour, the first quarter (5:00 to 5:15) of that hour was 207 watts, the next 15 minutes (5:15 to 5:30) was 251 watts, the next 15 minutes (5:30 to 5:45) was 257 watts, and the final 15 minutes (5:45 to 6:00) was an amazing 371 watts.

Based on this information, the low average power output really had nothing to do with the length, it was largely dictated by extremely low wattage in the middle of the race. In fact, there was so much floating in that middle section that over the course of the race, Floyd rode at zero watts for 84 minutes.

Essentially, these guys ripped the first hour, did nothing for the next 4 hours, and then ripped it for the last hour. Actually, they really put it down for the last 30 minutes. In fact, over the last category climb, Floyd sustained a power output of 474 watts for 5 minutes. That's only 4 watts less than the best he was able to hold on stage 9. So nobody was holding back on that climb.

Unfortunately, Floyd found himself a little too far back on that climb and got gapped. It won't happen again.

Table 1. Floyd's power data for stage 17.

*Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.*

| | Stage 17 | Tour Average | Min | Max |
|-------------------|------------------------------|--------------|--------------------------|------------------|
| Basic Stats | Duration (hrs:min) | 6:04 | 4:46 = 0:47 | 3:43 (S13) |
| | Distance (miles) | 148 | 123 ± 15 | 107 (S11) |
| | Average Speed (mph) | 24.3 | 26.2 ± 3.17 | 20.3 (S15) |
| | Perception of Effort (1-10) | 5 | 6.1 ± 1.8 | 4 (S2, S5) |
| | Average Cadence (RPM) | 86 | 85 ± 3.0 | 81 (S6, S11) |
| | Average Power (Watts) | 198 | 238 ± 25 | 206 (S2) |
| Peak Power | Work from Power (KJoules) | 4,326 | 4,097 ± 877 | 2,853 (S15) |
| | Work from RPE (KJoules) | 5,462 | 5,309 ± 2361 | 2,772 (S2) |
| | Stress to Strain Index | 1.26 | 1.28 ± 0.31 | 0.88 (S3) |
| | 5 sec (Watts) | 919 | 855 ± 76 | 757 (S11) |
| | 30 sec (Watts) | 597 | 563 ± 46 | 505 (S2) |
| | 1 min (Watts) | 538 | 484 ± 44 | 403 (S2) |
| | 5 min (Watts) | 474 * | 403 ± 56 | 308 (S2) |
| | 30 min (Watts) | 321 | 314 ± 50 | 249 (S3) |
| | 1 hour (Watts) | 271 | 286 ± 43 | 231 (S2) |
| | 2 hours (Watts) | 228 | 262 ± 34 | 227 (S2) |
| Zone Distribution | Zero Watts (% or min) | 23.1 % or 84 | 13.6 ± 4% or 39 ± 15.6 | 36 min (S8) |
| | Recovery (% or min) | 24.1% or 88 | 23.2 ± 5.9% or 65 ± 14.2 | 36 min (S11) |
| | Endurance (% or min) | 29.8% or 109 | 32.4 ± 1.9% or 92 ± 15.5 | 72 min (S2) |
| | Lactate Threshold (% or min) | 9.5% or 35 | 11.7 ± 4.8% or 34.6 ± 18 | 17 min (S2, S13) |
| | Race Pace (% or min) | 5.3% or 19 | 8.8 ± 3.5% or 26 ± 13 | 12 min (S2) |
| | Max (% or min) | 3.3% or 12 | 4.7 ± 1.1% or 13.6 ± 3.8 | 8 min (S2) |
| | Supra-Max (% or min) | 4.9% or 18 | 5.5 ± 1.1% or 15.4 ± 3.0 | 12 min (S11) |

©Allen Lim

Bicycling

Powerfeed: Exceeding The Limits

Stage 18: The culture of the Tour is to yearn for more than what was ever thought possible.

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Albi to Mende

Type: Climb, descend, climb, kinda flat, climb, rollers, hard climb to finish.

Distance: 189 km or 117.2 miles

Elevation Change: 868 meters or 2,848 feet gain (Average pt to pt grade = 0.46%)

Total Feet Climbed: 2,108 meters or 6,916 feet

Average Elevation: 644 ± 252 meters or 2,112 ± 827 feet above sea level

Minimum Elevation: 182 meters or 597 feet

Maximum Elevation: 1,084 meters or 3,557 feet

Weather: 77°F, clear, and 50% Humidity

Climbs:

1. Cote de la Bessede (Cat 4): 8.3 km at 2.9% beginning at 44.7 km point.
2. Cote de Raujolles (Cat 3): 2.7 km at 5.2% beginning at 96.3 km point.
3. Cote de Boyne (Cat 2): 9.2 km at 5.3% beginning at 121.3 km point.
4. Cote de Chabrits (Cat 3): 1.7 km at 7.1% beginning at 178.3 km point.
5. Cote de la Croix-Neuve (Cat 2): 3.1 km at 10.1% beginning at 184.4 km point

At a Glance:

Stage Placing: 20th

GC Placing: 9th

Time: 04:49:43 (12 min & 7 seconds down on winner Marcos Serrano)

Average Speed: 39.1 km/hr or 24.3 mph

Average Power: 222 Watts

Total Work: 3,859 Kjoules

Stress to Strain Index: 1.91

Tired:

The riders came across the line looking tired today. It's kind of strange how you don't notice the really subtle changes that occur each day. But this race has been wearing on the athletes from day one and for whatever reason you could just see it across the peleton as they came up the final climb today. They look noticeably thinner, haggard, quieter, and more direct with their actions - as if realizing that every movement is a precious use of energy.

The Climb:

And yet, on that last climb the very physics of movement have left me in awe. Today, after 17 days of racing, and 184.4 km into this stage, Floyd came up the 3.1 km Cote de la Croix-Neuve in 10 minutes and 16 seconds. At a grade of 10.1% he climbed that hill at a VAM or rate of ascent of 1823 meters per hour averaging 11.2 mph. That is like climbing just over one vertical mile (1.13 miles) or well past Boulder, CO from sea level in one hour. Based on the physical climbing rate, the math says that Floyd would have needed to produce 430 watts to climb that fast. In fact, isolated and riding alone, he averaged exactly that - 430 watts or 6.46 watts per kg. This is only 5 watts less than Floyd's highest 10-minute peak power of 435 watts that occurred on stage 8 during the last part of the final climb into Gerardmer, where riders were fresh and chomping at the climbing bit. If you think any of this is impressive then consider this, Floyd got dropped.

Possibilities:

A climb like today's is a real great one for us to analyze. Based on the power numbers from Floyd's PowerTap and the confirmation of those values against basic physics and math, we can get a nearly exact understanding of what Floyd would have needed to do to stay with the Yellow jersey group. To make up the approximately 30 second gap that the others had on Floyd over the top, Floyd would have needed to produce about 5% more power or 24 watts extra for a total of 454 watts. That would put him at a power to weight ratio of 6.78 watts per kg, a VAM of 1920 meters per hour, and a speed of 11.7 mph.

So there you have it. We just found another line and another goal. It is in my experience, what could be at the very limit of human possibility. But that's the culture of the Tour - to yearn for more than what was ever thought possible.

Table 1. Floyd's power data for stage 18.

*Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.*

| | | Stage 18 | Hour Average | Min | Max |
|-------------------|------------------------------|-------------|--------------------------|------------------|---------------|
| Basic Stats | Duration (hrs:min) | 4:50 | 5:46 ± 0:46 | 3:43 (S13) | 6:16 (S15) |
| | Distance (miles) | 117 | 123 ± 14 | 107 (S11) | 148 (S17) |
| | Average Speed (mph) | 24.3 | 26.1 ± 3.09 | 20.3 (S15) | 30.1 (S5) |
| | Perception of Effort (1-10) | 8.5 | 6.3 ± 1.9 | 4 (S2, S3) | 10 (S15) |
| | Average Cadence (RPM) | 91 | 85 ± 3.3 | 81 (S6, S11) | 91 (S18) |
| | Average Power (Watts) | 222 | 237 ± 24 | 206 (S2) | 285 (S11) |
| | Work from Power (KJoules) | 3,559 | 4,082 ± 940 | 2,853 (S13) | 5,620 (S13) |
| | Work from RPE (KJoules) | 7,388 | 5,525 ± 2334 | 2,772 (S2) | 11,286 (S15) |
| Peak Power | Stress to Strain Index | 1.91 | 1.32 ± 0.34 | 0.88 (S3) | 2.01 (S15) |
| | 5 sec (Watts) | 338 | 354 ± 73 | 257 (S11) | 465 (S8) |
| | 30 sec (Watts) | 337 | 361 ± 44 | 265 (S2) | 445 (S9) |
| | 1 min (Watts) | 495 | 484 ± 42 | 403 (S2) | 535 (S9) |
| | 5 min (Watts) | 457 | 407 ± 56 | 308 (S2) | 478 (S9) |
| | 30 min (Watts) | 354 | 317 ± 49 | 249 (S2) | 386 (S8) |
| | 1 hour (Watts) | 295 | 287 ± 42 | 231 (S2) | 359 (S11) |
| | 2 hours (Watts) | 247 | 261 ± 32 | 227 (S2) | 315 (S11) |
| Zone Distribution | Zero Watts (% or min) | 17.7% or 51 | 13.9 ± 4% or 40 ± 15.3 | 26 min (S8) | 34 min (S17) |
| | Recovery (% or min) | 21.6% or 63 | 23.1 ± 5.7% or 65 ± 14.6 | 36 min (S11) | 58 min (S17) |
| | Endurance (% or min) | 33.3% or 96 | 32.5 ± 1.9% or 93 ± 14.9 | 72 min (S2) | 115 min (S14) |
| | Lactate Threshold (% or min) | 9.5% or 28 | 11.6 ± 4.7% or 34.1 ± 18 | 17 min (S2, S13) | 49 min (S15) |
| | Race Pace (% or min) | 7.5% or 22 | 8.7 ± 3.4% or 26 ± 13 | 12 min (S2) | 50 min (S14) |
| | Max (% or min) | 5.3% or 15 | 4.8 ± 1.1% or 13.7 ± 5.7 | 8 min (S2) | 22 min (S8) |
| | Supra-Max (% or min) | 5.1% or 15 | 5.4 ± 1.0% or 15.3 ± 2.9 | 12 min (S11) | 24 min (S8) |

©Allen Lim

Bicycling

Powerfeed: Going Wireless

Stage 19: New equipment makes life easier for everyone

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Issoire to Le Puy-en-Velay

Type: Hard Rollers

Distance: 153.5 km or 95.2 miles

Elevation Change: 269 meters or 883 feet gain (Average pt to pt grade = 0.18%)

Total Feet Climbed: 1,491 meters or 4,892 feet

Average Elevation: 823 ± 192 meters of 2,702 ± 631 feet above sea level

Minimum Elevation: 358 meters or 1,175 feet

Maximum Elevation: 1,196 meters or 3,924 feet

Weather: 86°F and 60% Humidity, clear hot skies.

Climbs:

1. Cote des Gerbaudias (Cat 4): 6 km at 3.1% beginning at 17 km point.
2. Col de St. Eloy la Glaciere (Cat 3): 11.3 km at 3.2% beginning at 27.2 km point.
3. Col des Pradeaux (Cat 2): 11.4 km at 5.6% beginning at 56.6 km point.
4. Cote des Terrasses (Cat 4): 2.9 km at 3.7% beginning at 93.1 km point.
5. Cote de Malaveille (Cat 4): 2.9 km at 5% beginning at 104.6 km point

At a Glance:

Stage Placing: 71st

GC Placing: 9th

Time: 03:37:35 (4 min & 31 seconds down on winner Giuseppe Guerini)

Average Speed: 42.3 km/hr or 26.2 mph

Average Power: 223 Watts

Total Work: 2,911 Kjoules

Stress to Strain Index: 1.79

Sweet Freedom:

A couple of days ago, I ran out of clean underwear and had to make an important executive decision about my options here at the Tour. At the same time, we made a very similar executive level decision about Floyd's Power Tap, going somewhere we've never gone before - wireless. I wanted to keep it quiet for a few days. Get use to it and see how it worked before I made any long-term commitments to the idea. After the last three stages, I must say, the feeling is really incredible and the equipment has never worked better.

That equipment is the CycleOps PowerTap SL 2.4 which features coded digital technology that literally blasts Floyd's power data from hub to handlebar, no strings or wires attached. The computer also features a coded digital heart rate receiver and is USB downloadable. One of the perks of working with Floyd here at the Tour is being the first to access the latest and greatest in technology. Though the new wireless PowerTap has been working great for us now, it won't be available for purchase until early 2006.

On a side note, going wireless really got the team mechanics excited. They are as much about the functionality of the equipment the riders use as the aesthetic. Tearing off that wire, getting rid of that highly visible line on the backside of the bicycle, and showing off the clean tubes of the new BMC bike Floyd is riding with those oh so sexy low top Zipp 202 carbon rims, made them as giddy as a group of 8th grade school boys on a topless beach in Nice. Hmmm...maybe it's time to go home now.

Fast and Furious:

When Floyd crossed the line today, he had this look on his face. It's the kind of look that I'd imagine someone jumping off a plane while trying to figure out a really complex calculus problem would have. Wind blown, wide eyed, and thrilled - all while being utterly confused and perplexed. Though the numbers don't quite show it, today's stage into Le Puy-en-Velay was an impressive one that left Floyd in awe at the strength of his teammates and the peleton. At a rating of perceived exertion of 8 out of 10 for an average power of only 222 watts, today was definitely unique. There was just never any let up on a course that was much harder than it appeared on paper.

It wasn't so much the speed as the consistency of the pace and the never-ending undulations in terrain that made this stage hard. Based on the course profile map given to the riders by the Tour organization there was a total of 4,892 feet of total climbing listed on what was this year's second shortest road course. Every day, I've been asking Floyd to confirm that number using his own internal barometer, and he swears that there had to be at least 8,000 feet of climbing today. On the big mountain stages, his intuition was always within 500 feet of the actual, and so I have no reason to believe he's too far off. What the course maps just don't list are the hundreds of small little rollers that can add up and really make a day harder than expected.

Other factors that were major players in today's race included the heat and the accumulated strain of racing for almost 3 weeks. I wouldn't call it fatigue. I'd call it "when is this race ever going to end?" Floyd would call it "I just want to have an ice cold beer and a burrito in my own backyard." Whatever you want to call it, it didn't have a large effect on the power numbers from today. We had new lows for total time and distance and a surprising new low for Floyd's 5-second peak power (731 watts). Though there were also new lows for time spent at his endurance zone and supra-maximal zone, they really just reflect the lower overall time as the percent time spent in different zones is very similar to the Tour averages.

Health vs. Performance:

One thing I've been thinking a lot about lately as I watch these guys perform at super-human limits is literally how insane all of this is. Don't get me wrong, I'm as

GDC00832

impressed and inspired as the next guy at what these athletes are able to do. It's just that elite athletic performance is not necessarily synonymous with good health or wellness. As we get close to the end of this year's Tour, I think it's really important to emphasize that the numbers I've been broadcasting are truly bizarre and at the top end of what the human body should or can be asked to handle. As a point of reference, the Surgeon General defines "Physical Fitness" as, "the ability to carry out daily tasks with vigor and alertness, without undue fatigue, and with ample energy to enjoy leisure-time pursuits and to meet unforeseen emergencies." Furthermore, to be physically fit, the Surgeon General recommends that the average American, accumulate 1500 Kcal or Calories (approximately 1350 Kjoules) of physical activity per week. This means, that we should be doing things like walking, cycling, mowing the lawn, lifting weights, and jumping up and down to the extent that we burn 1,500 Calories doing so each week. It doesn't necessarily matter if we do that for 30-minutes each day, for 1-hour every other day, or at low or high intensities. It just matters that we do it.

As an example, a normal person will burn 100 Calories for every mile they walk. To walk a mile you need to take about 2,000 steps. So if you walked, 5 miles a day or 10,000 steps 3 times per week, you'd be at the recommended 1500 Calories. If you walked just over 2 miles a day, every day, you would be close to the Surgeon General's recommendation. The bottom line is, it doesn't take a lot of physical activity to be physically fit. Just walk more.

If you compare that 1,500 Calorie or 1350 Kjoule recommendation for cycling with Floyd's average day of 4,013 Kjoules here at the Tour de France, then you start to see how extreme this bike race is. At an average of 4,000 Kjoules a day, over a week, Floyd would have accumulated 28,000 Kjoules of exercise. That's almost 19 times more than what is recommended by the Surgeon General. Based on the numbers, I wouldn't exactly say what he's doing here is healthy.

Comparing how Floyd feels on most days relative to the Surgeon General's definition of physical fitness is another story. For the most part, when he's not racing his bike, he's not exactly the poster boy for carrying "out daily tasks with vigor and alertness." And if we're talking about "undue fatigue" then all of these guys are hurting. Finally, if anyone thinks that Floyd or any of his teammates has "ample energy to enjoy leisure-time pursuits and to meet unforeseen emergencies" at this year's Tour, then they are out of their mind.

In fact, being this physically fit is in no way healthy. The Surgeon General defines "Health" as the, "capacity to enjoy life and to withstand challenges. It is not merely the absence of disease." With that in mind, the question, "are we having fun yet" is really an important one. If I was racing, my answer would be, "maybe...if I wasn't on a mostly liquid diet, trying to burn more calories than a toaster oven left on high all day, all while navigating unknown roads at terrorizing speeds." Add to that the significant bone loss faced by many of these cyclists, the significantly impaired immune function from the stress of exercise and travel, their overdrawn hormonal systems, and the various musculoskeletal aches, pains, and injuries sustained during this race and healthy becomes the last word I'd use to describe a Tour rider.

Ultimately, the bottom line is you don't have to train for or ride in the Tour de France to be Physically Fit and Healthy. If anything not doing the Tour almost insures that you are physically fit and healthy. In the end, the value of something like the PowerTap technology and monitoring everything we monitor here at this race lies in helping to improve the quality of lives of every day individuals. The numbers here may be extreme, but tracking your own numbers and being disciplined about your own health and well-being shouldn't be. One thousand five hundred Calories a week. That's all it takes.

Table 1. Floyd's power data for stage 19.

| | Stage 19 | Tour Average | Min | Max |
|-------------------|------------------------------|--------------|--------------------------|------------------|
| Basic Stats | Duration (hr:min) | 3:38 | 4:52 ± 0:47 | 3:38 (S19) |
| | Distance (miles) | 99.2 | 121 ± 15 | 95.2 (S19) |
| | Average Speed (mph) | 26.2 | 26.1 ± 2.69 | 20.3 (S13) |
| | Perception of Effort (1-10) | 8 | 6.4 ± 1.8 | 4 (S2, S3) |
| | Average Cadence (RPM) | 89 | 85.5 ± 3.3 | 81 (S6, S11) |
| | Average Power (Watts) | 223 | 216 ± 24 | 206 (S2) |
| | Work from Power (Kjoules) | 2,911 | 4,013 ± 861 | 2,855 (S13) |
| Peak Power | Work from RPE (Kjoules) | 5,222 | 5,808 ± 2261 | 2,772 (S2) |
| | Stress to Strain Index | 1.79 | 1.35 ± 0.35 | 0.88 (S3) |
| | 5 sec (Watts) | 751 | 846 ± 77 | 731 (S19) |
| | 30 sec (Watts) | 538 | 560 ± 43 | 465 (S2) |
| | 1 min (Watts) | 454 | 482 ± 42 | 405 (S2) |
| | 5 min (Watts) | 389 | 406 ± 54 | 368 (S2) |
| | 30 min (Watts) | 323 | 317 ± 47 | 249 (S2) |
| Zone Distribution | 1 hour (Watts) | 271 | 286 ± 40 | 251 (S2) |
| | 2 hours (Watts) | 235 | 259 ± 32 | 237 (S2) |
| | Zero Watts (% or min) | 22.7% or 49 | 14.5 ± 4.5% or 41 ± 15.0 | 26 min (S8) |
| | Recovery (% or min) | 16.9% or 37 | 22.7 ± 5.7% or 63 ± 15.8 | 36 min (S11) |
| | Endurance (% or min) | 28.2% or 61 | 32.2 ± 2.1% or 91 ± 16.5 | 61 min (S19) |
| | Lactate Threshold (% or min) | 11.8% or 26 | 11.6 ± 4.5% or 33.5 ± 17 | 17 min (S2, S13) |
| | Race Pace (% or min) | 0.3% or 22 | 8.8 ± 4.3% or 25 ± 12 | 12 min (S2) |
| Zone Distribution | Max (% or min) | 5.9% or 13 | 4.9 ± 1.1% or 13.7 ± 3.5 | 8 min (S2) |
| | Supra-Max (% or min) | 5.2% or 11 | 5.4 ± 1.0% or 15.1 ± 3.0 | 11 min (S19) |
| | | | | 24 min (S8) |

©Allen Lim

Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

Bicycling

Powerfeed: The Classic

Stage 20: Tour riders are a class in themselves

By Allen Lim PhD

Here's the latest installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Saint-Etienne to Saint-Etienne

Type: Individual Time Trial

Distance: 55.5 km or 34.4 miles

Elevation Change: -3 meters or -10 feet loss (Average pt to pt grade = -0.01%)

Total Feet Climbed: 619 meters or 2,031 feet

Average Elevation: 625 ± 145 meters or 2,049 ± 475 feet above sea level

Minimum Elevation: 475 meters or 1,558 feet

Maximum Elevation: 834 meters or 2,031 feet

Weather: 79°F and 34% Humidity

Climbs:

1. Col de la Gachet (Cat 3): 5.7 km at 4.5% beginning at 34.3 km point.

At a Glance:

Stage Placing: 6th

GC Placing: 9th

Time: 01:13:48 (2 min & 2 seconds down on winner Lance Armstrong)

Average Speed: 45.1 km/hr or 28.0 mph

Average Power: 379 Predicted Watts based on speed, terrain, and drag

Total Work: 1678 Predicted Kjoules

Stress to Strain Index: 1.32

The Time Trial:

All I can say, is those guys went fast. After three weeks of some incredible racing, I am once again in awe at how much power these athletes can produce. With a 6th place finish in today's stage and a top ten GC almost all wrapped up, Floyd is really happy with his performance. He gave it his all and powered to an incredible finish. It's a really great start and an even better learning experience for the future. The bar has been set and we intend to reach the mark.

As for today's time trial, I did a bit of modeling again to predict what Floyd's power output needed to be for his time, the terrain, and the current conditions. I also decided to model a "what if." What if Floyd had the same time as the 5 riders ahead of him? What would his power be? For the 1:13:48 that Floyd posted, I believe his average power was approximately 379 watts. If Floyd had finished at the same time as Armstrong, his power would've been 410 watts - a whopping 8% better. For the riders just ahead of Floyd, the % difference was not as great with Basso, Julich, and Vinokourov finishing at modeled power outputs that for Floyd would've been 1%, 2%, and 3% better. Like Armstrong, Ulrich seems to be in a class of his own at 7% better than Floyd (Table 1).

| NAME | TIME | FLOYD'S POWER (W) FOR THEIR TIME | % DIFFERENCE IN POWER |
|---------------|---------|---|--------------------------|
| 1. ARMSTRONG | 1:11:46 | 410 | 8% |
| 2. ULRICH | 1:12:09 | 404 | 7% |
| 3. VINOKOUROV | 1:13:02 | 391 | 3% |
| 4. JULICH | 1:13:19 | 386 | 2% |
| 5. BASSO | 1:13:36 | 382 | 1% |
| 6. LANDIS | 1:13:48 | 379 | 0% |

Table 1. Based on Floyd's aerodynamic drag, body weight, and coefficient of rolling resistance as well as today's course profile and environment, the above table shows what Floyd's power output would be for his time and the time of the competitors ahead of him. Thus, the power listed and the % difference in power are not the power outputs for Floyd's competitors but the power outputs Floyd would have needed to do to match their time.

ITT Warm-Up Strategy:

I've been getting a lot of questions about Floyd's warm-up strategy for today's time trial. The reality is that his warm-up isn't really that complex, especially for a longer TT like the one here in Saint-Etienne.

©Allen Lim

Essentially, the mechanics will put Floyd's time trial bicycle on a trainer using a standard clincher-training wheel about an hour and a half before his scheduled start time. They'll put his disc wheel on right before he rolls out to the start.

For a time trial of this length, Floyd will only warm up for about 45-minutes, especially if it's warm out. He tries to finish his warm-up about 15-minutes before the start, so today he was on the bike about an hour before his start. During the warm-up Floyd tries to ride a fairly moderate pace, with maybe 3 to 4 intervals close to but not over his lactate threshold. One thing we've found is that if you go too hard during the warm-up you don't always produce the same kind of power output that you would if you went a little easier. The key is to really warm-up the legs and get comfortable riding the bicycle, not to kill yourself. For that reason, Floyd rarely does any high intensity surges or maximal efforts while warming up. It's all just moderate to somewhat hard with a few efforts just approaching hard. In a typical warm-up Floyd will average 275 watts, with a few efforts in the 350 to 400 watt range.

Today, Floyd was holding a cadence in the 85 to 95 rpm range during his warm-up. Jonathan Vaughters was hanging out with Floyd at the time and suggested that for his next time trial, Floyd try a much higher cadence of 110 to 120 rpm. I asked why, and Jonathan replied that he felt an important aspect to the warm-up was to get the neuromuscular system fired up and that pedaling at a faster than normal rate helped with this. Jonathan has a huge amount of knowledge and instinct in every aspect of cycling and we will definitely be trying his advice for the next TT.

During the warm-up Floyd tries to ride a fairly moderate pace, with maybe 3 to 4 intervals close to but not over his lactate threshold. One thing we've found is that if you go too hard during the warm-up you don't always produce the same kind of power output that you would if you went a little easier. The key is to really warm-up the legs and get comfortable riding the bicycle, not to kill yourself. For that reason, Floyd rarely does any high intensity surges or maximal efforts while warming up. It's all just moderate to somewhat hard with a few efforts just approaching hard. In a typical warm-up Floyd will average 275 watts, with a few efforts in the 350 to 400 watt range.

GDC00834

Today, Floyd was holding a cadence in the 85 to 95 rpm range during his warm-up. Jonathan Vaughters was hanging out with Floyd at the time and suggested that for his next time trial, Floyd try a much higher cadence of 110 to 120 rpm. I asked why, and Jonathan replied that he felt an important aspect to the warm-up was to get the neuromuscular system fired up and that pedaling at a faster than normal rate helped with this. Jonathan has a huge amount of knowledge and instinct in every aspect of cycling and we will definitely be trying his advice for the next TT.

The next strategy is to use an ice vest under his jersey during his warm-up. Essentially, it's a vest with an ice-like gel material embedded throughout that we keep in ice water before his warm-up. The ice vest helps to keep his torso really cold. The torso is essentially the radiator of the human body. When blood vessels dilate on your back, hot blood is brought to the skin surface where evaporating sweat can cool it off. By using the ice vest, we actually increase blood flow to his legs, because less blood needs to flow to the back for cooling. This effect actually can last for the first 10 to 30 minutes of the time trial as well, because the cold skin on his torso prevents his brain from regulating his body temperature at a higher than normal temperature which is typically the case during exercise. This means that more blood is allowed to flow to his legs because less blood is needed to keep his body cool. In addition, by keeping his torso cool, he doesn't have to sweat as much during his warm-up helping to keep him hydrated. What a lot of people don't realize is that one of the biggest rate limiting factors in time trial is not just having enough blood for oxygen delivery but having enough blood to keep the engine from over-heating. Once the body over heats it doesn't matter how much oxygen you can consume or how powerful your engine is, because everything just shuts off.

In summary, for Floyd's warm-up we try to get him on the bike an hour before the start. He rides at a moderate to hard intensity, but never goes in the red. We'd like to use a trainer with a more realistic road like feel so we can experiment with higher cadences. And finally, we do everything we can to keep him cool while he's getting hot.

*Dr. Allen Lim is an exercise physiologist and coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.*

Bicycling

Powerfeed: It's Only The Beginning

Insight into the most challenging endurance event in the world

By Allen Lim PhD

Here's the last installment of our Powerfeed coverage, with detailed reports on Floyd Landis' performance from his power coach, Allen Lim from data collected off the CycleOps Powertap on Landis' bike. Need a **glossary**?

Route: Corbeil-Essonnes to Paris Champs-Elysees

Type: Flat Stage

Distance: 144.5 km or 89.6 miles

Elevation Change: -25 meters or -82 feet loss (Average pt to pt grade = -0.02%)

Total Feet Climbed: 304 meters or 997 feet

Average Elevation: 70 ± 42 meters or 231 ± 138 feet above sea level

Minimum Elevation: 40 meters or 131 feet

Maximum Elevation: 172 meters or 564 feet

Weather: 66°F and 94% Humidity with a light drizzle

Climbs:

1. Cote de Gif-sur-Yvette (Cat 4): 1.5 km at 5.5% beginning at 56 km point.

At a Glance:

Stage Placing: 91st

GC Placing: 9th

Time: 03:40:57 (Same time as winner Vinokourov)

Average Speed: 39.2 km/hr or 24.3 mph

Average Power: 164 Watts

Total Work: 2,174 Kjoules

Stress to Strain Index: 1.22

Today's Stage:

As expected, **today's stage** was one of the easiest of the Tour, scoring the lowest average power output (164 watts), the lowest total work based on power (2,174 Kjoules), the lowest rating of perceived exertion (4), the lowest total work based on perception of effort (2,651 Kjoules), the shortest distance (89.6 miles), and nearly the shortest time (3:41vs. stage 19's 3:38). In addition, today Floyd spent the least amount of time at all intensity zones except zero watts and his recovery zone. It was a day, that I'd take the risk of saying I might have even been able to finish. Well, maybe if I hadn't spent my last three weeks sitting in a car.

While today's stage was the easiest, it wasn't all a walk in the park. Over the last 30 minutes, the power did come up to an average of 263 watts during the finishing circuits. Floyd spent most of that time floating and trying to stay out of trouble. As beautiful of a stage as it was, I guess it was a bit anticlimactic from a power perspective. That said, over the course of this Tour we've gained some incredible insight on an event that I truly believe is the most challenging endurance event in the entire world.

Totals:

Over the last three weeks Floyd raced his bike (including time trials) a total of 86 hours and 26 minutes, give or take a handful of seconds. That's about 29 hours a week, which is close to what he was averaging in the two months before the Tour. Over that time, he rode a total of 2,116 miles. In doing so, he took approximately 443,922 pedal strokes.

At an average power output of 232 watts over those three weeks, he did a total of 70,914 Kjoules of work, burning somewhere around 74,460 Kcals. That's equal to about 143 large McDonalds Fries, 133 Big Macs, or 372 Krispy Kreme donuts. And while that might sound like a lot, compared to the energy we actually use back home, it's barely even a hiccup in the grid. For example, for the total amount of energy that Floyd expended in this year's Tour de France, he could have kept the state of California powered during peak energy usage for only 22.69 seconds, give or take a second. That means if we were able to harness all of the energy from the entire peloton from all three weeks, we might have kept California going for about 1 hour and 11 minutes.

Finally, looking back at where Floyd spent his energy, the short story is that he spent 30% at or above his lactate threshold and 70% below it. In a sense, 30% of his total time was spent at intensities he'd consider hard to all out, and 70% of his time cruising.

In the end, however, we believe it was the time he didn't or couldn't spend at supra-maximal intensities that had the biggest impact on Floyd's Tour. At a total of 234 minutes or 3.9 hours at or above 500 watts, we think that if Floyd was able to spend 10 more minutes (4% more) at that top end, he might have been on the podium.

In any case, we learned and experienced more than we ever expected and couldn't be happier about getting through and being a part of this year's Tour. Thanks for taking the time for reading and learning with us.

Table 1. Floyd's power data for stage 21.

Table 2. Total values for the entire Tour de France, 2005.

Dr. Allen Lim is an
exercise physiologist and

GDC00836

| | Stage 21 | Tour Average | Min | Max |
|-------------------|------------------------------|--------------|----------------------------|-----------------|
| Basic Stats | Duration (hr:min) | 3:41 | 3:42 ± 1:07 | 3:38 (S19) |
| | Distance (miles) | 89.6 | 115 ± 25 | 89.6 (S21) |
| | Average Speed (mph) | 24.3 | 26.0 ± 2.92 | 30.3 (S15) |
| | Perception of Effort (1-10) | 4 | 6.3 ± 1.9 | 4 (S2, S3, S21) |
| | Average Cadence (RPM) | 88 | 85.0 ± 3.23 | 81 (S6, S11) |
| | Average Power (Watts) | 164 | 232 ± 29 | 164 (S21) |
| | Work from Power (KJoules) | 2,174 | 3,911 ± 941 | 2,174 (S21) |
| | Work from RPE (KJoules) | 2,651 | 5,348 ± 2294 | 2,651 (S21) |
| | Stress to Strain Index | 1.22 | 1.34 ± 0.34 | 0.88 (S3) |
| | 200 (S15) | | | 200 (S15) |
| Peak Power | 5 sec (Watts) | 772 | 841 ± 77 | 731 (S19) |
| | 30 sec (Watts) | 477 | 555 ± 47 | 463 (S4) |
| | 1 min (Watts) | 434 | 479 ± 42 | 403 (S2) |
| | 5 min (Watts) | 316 | 400 ± 57 | 308 (S2) |
| | 10 min (Watts) | 263 | 314 ± 48 | 249 (S2) |
| | 1 hour (Watts) | 240 | 283 ± 41 | 231 (S2) |
| | 2 hours (Watts) | 193 | 255 ± 35 | 193 (S21) |
| | 315 (S11) | | | 315 (S11) |
| Zone Distribution | Zero Watts (% or min) | 23.4% or 52 | 15.0 ± 4.9% or 41.2 ± 14.2 | 26 min (S5) |
| | Recovery (% or min) | 35.3% or 78 | 23.5 ± 6.4% or 63.9 ± 15.7 | 56 min (S11) |
| | Endurance (% or min) | 25.6% or 57 | 31.8 ± 2.6% or 86.3 ± 18.0 | 57 min (S21) |
| | Lactate Threshold (% or min) | 5.6% or 12 | 11.3 ± 4.6% or 32.2 ± 17.3 | 12 min (S21) |
| | Race Pace (% or min) | 3.9% or 9 | 8.5 ± 3.4% or 24.2 ± 12.4 | 9 min (S21) |
| | Max (% or min) | 2.6% or 6 | 4.7 ± 1.2% or 13.2 ± 4.0 | 6 min (S21) |
| | Supra-Max (% or min) | 3.7% or 8 | 5.3 ± 1.1% or 14.6 ± 3.4 | 8 min (S21) |
| | 24 min (S8) | | | 24 min (S8) |

©Allen Lim

| | Totals |
|------------------------------|-----------------------|
| Duration (hrs:min) | 86 hrs 26 min |
| Distance (miles) | 2,116 miles |
| Average Cadence (RPM) | 443,922 Pedal Strokes |
| Work from Power (KJoules) | 70,914 KJoules |
| Work from RPE (KJoules) | 96,886 KJoules |
| Zero Watts (% or min) | 659 |
| Recovery (% or min) | 1,023 |
| Endurance (% or min) | 1,416 |
| Lactate Threshold (% or min) | 515 |
| Race Pace (% or min) | 387 |
| Max (% or min) | 211 |
| Supra-Max (% or min) | 234 |

©Allen Lim

has focused primarily on advising **Floyd Landis** bringing a unique blend of practical, scientific, and philosophical insight to Floyd's Tour de France preparation.

coach with **Thrive Health Fitness Medicine**. He received his doctorate at the University of Colorado at Boulder where his research focused on health and human performance with a specific emphasis on optimizing cycling performance with the use of cycle mounted power meters. Over the last decade, Dr. Lim has helped guide a number of teams and individual cyclists to collegiate, national, world, and Olympic medals. This last year, however, he

Bicycling

Powerfeed PreTour: The Tour From A Human Perspective

A dedicated scientist takes some time out from the numbers to get in touch with the true spirit of the Tour de France

By Allen Lim PhD

It's been over a year and a half now since I first met Floyd Landis. At the time, I was close to finishing grad school. But instead of feeling optimistic about the future, I was bitter, burnt, and broke. My only plan was to leave Boulder and head back to Los Angeles. I figured I could live with my mom for a while - keep her company and help out around the house. Looking for a career or a future was the last thing on my mind. I just wanted to ride my bike, take some time to figure things out, and just be.

Before I had time to pack up, I got a call from David Cathcart, a close friend who also happened to be the director of marketing for CycleOps. He called to tell me that they had just signed a deal with Floyd who was leaving US Postal to ride for Phonak. David wanted to know if I'd be interested in helping Floyd out with a camp he was putting on. Both Floyd and David would be coming through Boulder and they were interested in meeting to talk details. So a week later I found myself meeting Floyd at a local coffee shop listening to an anthology of unsolicited and unfiltered rants that blasted with the energy of a broken fire hydrant. I thought to myself, "are you kidding me, this guys more bitter than I am..., and why am I so bitter."

After Floyd's camp, Robbie Ventura, Floyd's coach asked if I would help with Floyd's training and power analysis. Compared to Floyd, Robbie was like a bouncy-ball that had just been launched from a 20-story building. Robbie beamed with so much positive energy that it was almost inconceivable that he and Floyd could co-exist in the same space. We sat there in Floyd's living room as Robbie translated for us, rallying the troops like we were getting ready for a field trip to the moon. But it wasn't until I heard Floyd say, "hey Al, I think I can win the Tour. What do you think?" that I really started to pay attention. It was the first time in a long time.

By early May, I was on a flight from Denver to Barcelona heading for Girona, Spain to help a former Mennonite from Pennsylvania take on the biggest race in the world. I arrived in Girona worked and with no bags to find Floyd about to feast on a dinner of cereal and gummy bears. I looked at him and he looked at me. I shook my head and he shrugged his shoulders and then I shyly asked, "Is there a market around here?" An hour later we had a dinner fit for Kings or maybe the odd couple - two eggs over pasta with some Spanish version of Ragu on top. It was a better alternative. We ate on the couch in Floyd's dorm size apartment and watched Star Wars, waxing philosophic about the force, dark side, and Luke Skywalker's inherent belief that he was destined for something bigger than the desert of Tatooine.

Maybe it was the setting, but I felt like a freshman in college all over again -- the same fear, hope, and naive confidence about the future and all of its unknown possibilities. For the first time in a long time, I also missed home. It's that feeling you get when you know you have to go but don't want to. I didn't know the language, I didn't know where I was, and I didn't have my luggage. But my bags eventually arrived, I started picking up the local lingo, and over the next month we both began figuring it out. We set up camp in the Pyrenees and I watched Floyd train harder than I've ever seen anyone train in my entire life. Unfortunately, we came short of our expectations at the Tour. It was humbling but I think we both came home better.

This year, things have been quite different. I took a job with Slipstream Sports as the full time sport scientist for the TIAA-CREF Cycling Team run by Jonathan Vaughters. All the hype about "what's next" at the end of last season was driving me crazy and I thought I'd just go help Jonathan with "what's now." At the same time, Floyd was having the early season of his life and with all of the new found attention, the team played a much more active role in his preparation. For the first time, Floyd was running the show and had the respect and care he deserved. So beyond time together in Mallorca for training camp, some wind tunnel testing, the races back at home and the Dauphine, we didn't get the same quality time roughing it in the Pyrenees like we did last year. Coming back to the Tour this year, I've found myself sentimental for what seems like a whole other era. Perhaps it's that sentiment, that has me looking ahead at this year's Tour with a different perspective. It's the human perspective on the facts and figures that I slaved over at last year's Tour. Although my job this year includes daily power analysis for Floyd and additional teammates who will be riding the PowerTap, I've come to learn over the years that it's not the numbers that makes you good. There's so much more. So in between the average power outputs, the time spent in different zones, peak power outputs, and calculations of ascent rates, I want to try and share more of the human and daily stories that drive this team and all of the individuals associated with it.

Today, Floyd, Robbie Ventura, and J  se Bartholomew who will be my power analysis assistant went for a ride to check out the prologue course. As we started rolling out, Floyd started to sing some tunes by Kid Rock. It put a smile on all of our faces. I looked at Floyd and he laughed and I thought, "wow, this guys going to win the Tour de France."

Bicycling

Powerfeed Prologue: The Average Guy

Riding the prologue course with Floyd Landis puts power in perspective

By Allen Lim PhD

Everyone always asks if I get to ride a lot while I'm at the races and my basic answer is, never. But this year, Jesse Bartholomew, Robbie Ventura, and I all decided to bring our bikes to the Tour. Although our job is to help Floyd ride as fast as he can each day, we figured that between the three of us, we could at least motivate each other to find the occasional hour to get out on the bike. So when Floyd asked us to meet him to pre-ride the prologue course again this morning, we immediately began making plans for the big day.

All week long we've been playing odd-ball games. From naming lines in movies to seeing who can lose the most weight after using the bathroom, our techniques for passing the time span from benign to pretty stupid. And despite the fact that Floyd is a world-class athlete leading a team in the Tour de France, we still like to imagine that we can compare or even compete with him. Basically, when you get us all together we're like a bunch of kids trying to trump the other with the latest "high score." So this morning the goal was to see who could put out the biggest one minute power effort on the prologue course. And with oddly deluded reasoning we thought we could make things fair by just normalizing to body weight. On the elevator ride down from our rooms, we all made our "your mama" predictions. Floyd laid claim to 700 watts and we all just swaggered with confidence like that was a little number. We played it cool, thinking, "whatever man, bring it on."

We took the first lap pretty easy, checking out the best lines through the corners and looking for the smoothest asphalt to cruise over. Floyd got comfortable in his time trial position, letting other riders and teams fly past him as he settled into an easy rhythm, chatting it up with us and humming his favorite tunes. I was feeling good and looking forward for things to get started. Unfortunately, none of us really coordinated exactly when or where we were going to do our effort. And as we came through on the second lap, Floyd decided to open it up a bit. Before we knew what hit us, we were scrambling like we stole something, fighting for Floyd's wheel as if our lives depended on it. I won that battle, but soon realized that there's pretty much zero draft behind Floyd when he's in his time trial position. The first thing I thought was, "oh man, I am screwed." Actually, the person who got screwed was Jesse as I gapped the guacamole out of him. Jesse claims that he wasn't fighting for Floyd's wheel cause, of course, in his words, "there's no freaking draft behind that guy." To his credit, Jesse closed the gap bringing Robbie in tow. I was done, but having got in at least a one-minute effort, I figured I was good for the day. Robbie then came around Jesse giving him some protection and while they both get an A for effort, Floyd eventually sawed them off. I just cruised and waited for him to lap me, which I figured would take less than 8 minutes. After downloading the data, it was confirmed that our best 1-minute effort did indeed occur in that initial dogfight for Floyd's wheel. And, of course, Floyd kicked all of our butts at an estimated 9 watts per kg. I came in at a respectable 6.9 watts per kg, while Robbie and Jesse were tied at 5.8 watts per kg.

Later this afternoon, Floyd's luck wasn't quite as good. While cruising around the start house he got a deep cut in his rear wheel. He decided to play it safe and let the mechanics change it. Unfortunately, his disc wheel doesn't have a quick release and the whole thing turned out to be more of a hassle than expected. Some time was lost at the start, but having still finished in the top ten, we were all glad that he played it safe instead of sorry.

To try and shed some perspective on the day's results and weed out the bad luck at the beginning, I set to work on some mathematical modeling to calculate out the estimated power outputs of his competitors and to also calculate the virtual power outputs for Floyd had he actually won the race. In the top chart attached below, you can see what his competitors achieved and what Floyd actually achieved based on his real velocity (not time). Also in the chart, you can see what Floyd would've had to do to match the winning time. Essentially the difference in time amounts to a 4-watt difference in power output. On today's course that extra 4-watts can come from anywhere. As an example, we estimate that Floyd had to coast for 30 seconds today through the corners. Had he only coasted 26 seconds he would have gained those 4 extra watts. In any case, the math and the actual result give us a lot of confidence. Things happen and we're not losing any sleep over it. Instead we're looking ahead to stage 1 as we've all got bets on how low Floyd can keep his average power in tomorrow's flat stage. In the meantime, Robbie, Jesse, and I will be scheming for more points.

ESTIMATED POWER OUTPUTS FOR TOP 10 IN PROLOGUE:

| Name | Time | | Speed | | Moving Power | Peddalling Power |
|----------------------|------|-------|-------|-------|--------------|------------------|
| | Min | Sec | km/hr | mph | Watts | Watts |
| 1 Thor Hushovd | 8 | 17.00 | 51.43 | 31.89 | 487 | 519 |
| 2 George Hincapie | 8 | 17.73 | 51.35 | 31.84 | 460 | 490 |
| 3 Virtual Floyd | 8 | 18.26 | 51.30 | 31.81 | 435 | 463 |
| 4 Dave Zabriskie | 8 | 21.21 | 51.00 | 31.62 | 434 | 462 |
| 5 Sebastian Lang | 8 | 21.80 | 50.94 | 31.58 | 460 | 490 |
| 6 Alejandro Valverde | 8 | 21.92 | 50.92 | 31.57 | 419 | 445 |
| 7 Stuart O'Grady | 8 | 21.93 | 50.92 | 31.57 | 440 | 468 |
| 8 Michael Rogers | 8 | 23.30 | 50.78 | 31.49 | 453 | 482 |
| 9 Paolo Savoldelli | 8 | 25.02 | 50.61 | 31.38 | 425 | 452 |
| 10 Real Floyd | 8 | 26.26 | 50.49 | 31.30 | 425 | 451 |

ESTIMATED POWER OUTPUTS FOR FLOYD IN EACH DIFFERENT TOP 10 PLACINGS:

| Name | Time | | Speed | | Moving Power | Peddalling Power |
|---------------|------|-------|-------|-------|--------------|------------------|
| | Min | Sec | km/hr | mph | Watts | Watts |
| 1 Floyd 1st | 8 | 17.00 | 51.43 | 31.89 | 438.4 | 467 |
| 2 Floyd 2nd | 8 | 17.73 | 51.35 | 31.84 | 436.6 | 465 |
| 3 Floyd 3rd | 8 | 18.26 | 51.30 | 31.81 | 435.3 | 463 |
| 4 Floyd 4th | 8 | 21.21 | 51.00 | 31.62 | 428.3 | 456 |
| 5 Floyd 5th | 8 | 21.80 | 50.94 | 31.58 | 426.9 | 454 |
| 6 Floyd 6th | 8 | 21.92 | 50.92 | 31.57 | 426.6 | 454 |
| 7 Floyd 7th | 8 | 21.93 | 50.92 | 31.57 | 426.6 | 454 |
| 8 Floyd 8th | 8 | 23.30 | 50.78 | 31.49 | 423.4 | 450 |
| 9 Floyd 9th | 8 | 25.02 | 50.61 | 31.38 | 419.5 | 446 |
| 10 Floyd 10th | 8 | 26.26 | 50.49 | 31.30 | 416.7 | 443 |

Created By Allen Lim

Bicycling

Powerfeed Stage 1: Break it Down

When you sit down and try to figure out how to win the Tour de France, the first thing you realize is that you can't really look at it as a three-week race

By Allen Lim PhD

Our guess is that at this year's Tour there will, hopefully, only be eight days in the pain cave, thirteen days at medium heat, and three days just riding bikes. Of those eight hard days, two will probably occur in the first week (Sun to Sat), two in the second week, and four in the last week. The only problem with those four days in the last week is that by the time you get that deep into the cave, everyone's flashlight is dimming and it's unlikely you'd find a spare battery waiting for you...unless, somebody's already been down that cave, but that's a totally different story.

Of the thirteen medium or average days, seven will likely occur in the first week, four in the second week, and one in the last week. Finally, of the three days of just riding, zero will occur in the first week, one will occur on the first rest day in Bordeaux at the beginning of the second week, and two will occur in the last week - the rest day in Gap and the ride into Paris.

Long story short, we're all just trying to take it easy right now. And that includes the riders. With that in mind, there's really not that much to talk about with respect to the power data, except for the fact that it's taking us more time to break everything down each evening. Unlike last year, where Floyd was the only one on the team who rode the PowerTap each day, the "I want to be cool too" factor has given us four additional riders who will be on power meters this year - Koos Moerenhout, Robbie Hunter, Victor Hugo Pena, and Nicolas Jalabert. In addition, last year nobody on the team except for Floyd was interested in the data, but this year Jesse and I are spending our evenings writing up summary reports for the directors, doctors, riders, and our chef Gisbert (he just likes to be in the know).

Preparing for these reports has been difficult because they need to be detailed enough to give everyone an exact picture of everything that is going on with each rider and the team, while also serving as this team's first introduction to a concerted monitoring process. So deciding on exactly what power data and accompanying information to give the team has been a challenge. Here's what we've settled on:

- 1. Basic stage and general classification information.** This includes finishing time, place, time gaps, distance and speed.
 - 2. Weather Information.** Temperature, humidity, wind, radiation, and heat index measures.
 - 3. Race Notes.** A short summary of what happened and how individual riders felt about the day. This is my favorite thing to read back on when I look at old training diaries.
 - 4. Average Power or Stress.** This is broken down into power when the bicycle is moving versus the power produced when the rider is actually pedaling (i.e., excluding coasting time). Power data is given in absolute terms as a "Watt" and normalized to body weight in "Watts per Kg of Body Weight." In very simple terms, the average power is the average of intensity of the race.
 - 5. Strain.** This is basically a perceived rating of how hard or intense the riders felt the race to be and a measure of the rider's heart rate response - a physiological measure of the intensity of the race. This information is extraordinarily important because with this information and the power information, we have what actually happened from a mechanical or physical perspective (power) and how the athletes responded, from a psychological and cardiovascular perspective.
 - 6. Work Done.** When you take the average intensity and factor in the total time of the ride, you get the total work done or energy expended. Because we draw intensity measures from power, perceived exertion, and heart rate we get work scores for all three that we then normalize to Kjoules - a mechanical measure of work versus the Kcal, which is a thermal measure of work.
 - 7. Work Ratios.** All things being equal, the work done measured with the power meter should match the work measured by perceived exertion (RPE) and heart rate. Generally speaking, if the rider thinks that the ride was a lot harder or easier than it actually was, then the ratio between work measured by power (Stress) vs. work measured by heart rate or RPE (Strain) rises or falls. Looking at these ratios is a lot like looking at the actual temperature versus the wind chill factor or heat index. There's what's actually happening and how you're responding to it and it's that very simple dose-response paradigm that is at the heart of proper training.
 - 8. Power Distribution.** We look at how the power output was distributed each day in three ways. The first is time not pedaling. It's kind of a side competition, but in actuality it's a great measure of how much rest a rider is getting. The second way we look at power distribution is normalized to body weight in increments of 1 watt per kilogram of body weight. From the team's perspective, this will probably be the most important measure on the make or break days as there is an absolute power to weight that a rider is going to have to be able to do to stay with the leaders or win on the climbs. More importantly, if you aren't spending the same amount of time in different power to weight zones as the leaders, you probably aren't near the lead. And if you are spending the same time, as the leaders in these zones but aren't in the lead, you're either a wicked strong super domestique helping a team leader or just a really bad bike racer.
 - 9. Peak Power Output.** We list out the best power output for different time frames ranging from 5 seconds to 1 hour as well as the distance they occurred at so the riders can reference their road maps and see when they pounded big efforts.
 - 10. Surges.** This is a count of how many times a rider spikes above 6 watts per kg, 8 watts per kg, and 10 watts per kg. It's really indicative of how many attacks to place and how unsteady the race was. Generally speaking, training looks like a calm lake compared to the turbulence of a race.
 - 11. Hydration and Energy Status.** We weight the riders before and after each stage and take a count of all the bottles and food they drink. This allows us to figure out their sweat rates, sweat loss, and help give the riders feedback about their drinking needs. By combining this info with the "Work Done" and "Weather" info we can make future predictions about their fluid needs based on the race profile and weather.
 - 12. Climbs and Special Features.** In this section we break down exactly what happened on a particular climb or "special" moment. And there are always a lot of special moments. Anyway, we do a mathematical estimate of what should've happened and then compare that with what actually happen. Since all of this analysis is performed relative to terrain, the riders have a deeper frame of reference for those numbers as they all orient to geography when they think about their race. It's never, "when I was doing my best 5 minute effort in my E2 X-Beta Alpha zone." Instead it's something like, "when I was putting the smack down after the left when we passed the van down by the river."
- Despite the depth of these reports, what I'm trying to get at is that during the first week of this race, what we're really hoping to show the riders, and what we are expecting, is that things are pretty normal. Not too easy, but not too hard. And so, there's not too much to talk about right now, despite the fact that I'm way over my word limit. Right now our major focus is to just have the riders be aware of what exactly is going on. To help them tune their own sense of feel. This focus will change as we hit more challenging stages.

Attached is Floyd's report. If you don't want to look at it, all you need to know is he did an average of 205 watts while moving, 250 while pedaling, spent 45 minutes coasting, burned 3075 Kjoules or 3167 Kcals, drank 10 bottles of water, lost only 0.4 kilograms of body weight, and thought the day was not easy, but not hard, but probably more easy than hard. I'm sure that makes perfect sense.

**LE TOUR 2006: DAILY ANALYSIS REPORT****Stage 1: Strasbourg to Strasbourg, 184.5 km****Sunday, July 2nd****Floyd Landis**Temp: 82 °F / 28 °C Humidity: 41 % Heat Index: 82 °F / 28 °C**Stage Results:** 184.5 km 44.3 km/hr 27.5 mph**GC Results:** 191.6 km ridden 44.5 km/hr 27.6 mphPlace: 46 Time: 4 hrs 10 min 0 sec Gap: 0 min 0 secPlace: 9 Time: 4 hrs 18 min 26 sec Gap: 0 hrs 0 min 9 sec**Race Notes:** Today the guys just cruised. It was on the warm side and the racing was a bit nervous but all in all it was a pretty chill day for all involved.**Power, RPE, Heart Rate, Work, RPE vs. Power, HR vs. Power**

| | | Power when Moving | | Power when Pedaling | | Strain | | Work or Stress in Kilojoules From: | | | RPE | |
|-------|----------|-------------------|----------|---------------------|----------|--------|-----|------------------------------------|-------|-------|---------|---------|
| | | Watts | Watts/kg | Watts | Watts/kg | RPE | HR | Power | RPE | HR | Pwr | HR |
| Rider | Today | 205 | 2.92 | 250 | 3.56 | 3.5 | 123 | 3,075 | 3,277 | 3,141 | 1.07 | 1.02 |
| | Tour Avg | | | | | | | | | | =DIV/0! | =DIV/0! |
| | Tour Min | | | | | | | | | | | |
| | Tour Max | | | | | | | | | | | |
| Bike | Today | | | | | | | | | | | |
| | Tour Avg | | | | | | | | | | | |
| | Tour Min | | | | | | | | | | | |
| | Tour Max | | | | | | | | | | | |

Prepared By ©Allen Lim

Bicycling

Powerfeed Stage 3: Developing A Sense Of Self

There's no context for any of the power values without a sense of what an athlete feels

By Allen Lim PhD

How'd you feel today? It's probably the most common question that the riders get asked when they roll in each day. How was it? All good? How'd it go? Cava?

Despite all of the technology we unleash on these guys and all the analysis we do each day, the bottom line is still feel. As a professional athlete at the Tour or a weekend warrior at a local triathlon, there is no substitute for developing a deep and intuitive sense of self. Not that these guys are ultra-sensitive touchy feely types, but when you ask them how they feel, they all have a pretty extensive vocabulary for it.

When I asked Victor Hugo Pena how he felt the other day, his first reaction was, "Oh ya, it was hard." Which was quickly qualified by an explanation that it was only hard because the race is just getting started this week and the legs are still "opening" up. That was further clarified by an explanation about how it wasn't actually hard relative to how hard it will get or can get or actually is or could be or should be. A couple of hand gestures and discussion points later, it all boiled down to, "So ya, it wasn't that hard. Maybe a 4, you know, compared to other races. But it was hot, so more like a 4.5." I was kind of spacing at this point, thinking about how this discussion might go for Victor "The Shark" Pena in the bedroom. That turned into a laugh and I replied, "Cool, she'll give you, I mean, we'll give you a 4.5."

We use a simple 1-10 rating of perceived exertion scale, popularized by Carl Foster, who demonstrated that taking a simple 1 to 10 measure of perceived intensity and multiplying it by time in minutes, gives an extraordinarily robust measure of an athlete's training load – a measure of load that could be used across different sports and which is predictive of changes in performance during the season. For me, this is probably one of the most important things we monitor each day. The bottom line is that there's no context for any of the power values without a sense of what an athlete feels. In fact, we're not using these numbers to tell these guys what to do. We're using these numbers so that they can develop a deeper understanding of their personal responses relative to an objective measure of their performance – its just feedback. And feedback is really the most important thing. Knowing that you need to hold a bazillion watts per kg for 30 minutes to win the Tour is one thing. Knowing that you feel great doing it. That's pretty cool.

Anyway, it's the Fourth of July and Floyd wants to blow something up, so I need to run and help celebrate. All is good with the team though Victor lost some time today. I haven't talked to him about it yet, but I'm sure the explanation will be good.

| Name: <u>Floyd Landis</u> | | Stage: <u>3. Pink-Sau-Au-dre to Villard, 276.5 km</u> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--------------------------------|---|---|------|------------------|--------------------------------|----------------|--------------|--------|--------------|----|----------------|--------------|-------------------|--|---------------------|--|------------------|------------|--------------|-------|--------|--------------|------|------|-------|------|--------------|--------------|-----------|--|--|---------|
| Stage Place: <u>44th</u> | Time: <u>4:57:49</u> | Date: <u>Tuesday, July 26, 2005</u> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| GC Place: <u>7</u> | Time: <u>14:52:22</u> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | <table><tr><th colspan="6">Time (Min) Distributed Between</th><th>Best Power (W)</th><th># of Attacks</th></tr><tr><th colspan="2">Average Power (W)</th><th colspan="2">Work (KJoules) Done</th><th>Easy to Moderate</th><th>Kinda Hard</th><th>Greater Than</th><th>5 min</th><th>30 min</th><th>Greater Than</th></tr><tr><th>Mean</th><th>Peak</th><th>Pacer</th><th>Prod</th><th>On Very Hard</th><th>On Very Hard</th><th>Very Hard</th><th></th><th></th><th>10 W/Kg</th></tr></table> | | | | Time (Min) Distributed Between | | | | | | Best Power (W) | # of Attacks | Average Power (W) | | Work (KJoules) Done | | Easy to Moderate | Kinda Hard | Greater Than | 5 min | 30 min | Greater Than | Mean | Peak | Pacer | Prod | On Very Hard | On Very Hard | Very Hard | | | 10 W/Kg |
| Time (Min) Distributed Between | | | | | | Best Power (W) | # of Attacks | | | | | | | | | | | | | | | | | | | | | | | | | |
| Average Power (W) | | Work (KJoules) Done | | Easy to Moderate | Kinda Hard | Greater Than | 5 min | 30 min | Greater Than | | | | | | | | | | | | | | | | | | | | | | | |
| Mean | Peak | Pacer | Prod | On Very Hard | On Very Hard | Very Hard | | | 10 W/Kg | | | | | | | | | | | | | | | | | | | | | | | |
| Today | 327 | 270 | 5960 | 4000 | 185.9 | 73.4 | 27.7 | 490 | 282 | 41 | | | | | | | | | | | | | | | | | | | | | | |
| | Total Average | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 299 | 263 | 5670 | 3508 | 192.8 | 64.23 | 33.79 | 195 | 290 | 34 | | | | | | | | | | | | | | | | | | | | | | |
| | Meanmax | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Maxmax | 156 | 250 | 5978 | 1115 | 515.9 | 49.1 | 24.2 | 368 | 252 | 22 | | | | | | | | | | | | | | | | | | | | | | |
| | Total Average | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 232 | 276 | 5962 | 4117 | 329.8 | 58.9 | 37.7 | 409 | 329 | 42 | | | | | | | | | | | | | | | | | | | | | | |
| | Meanmax | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Today | 214 | 275 | 3856 | 254 | 102 | 72.8 | 39 | 410 | 208 | 32 | | | | | | | | | | | | | | | | | | | | | | |
| | Total Average | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 205 | 262 | 3634 | NA | 250.5 | 64.4 | 31.7 | 356 | 279 | 29 | | | | | | | | | | | | | | | | | | | | | | |
| | Meanmax | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Maxmax | 146 | 249 | 3918 | NA | 515.5 | 42.8 | 21.3 | 379 | 331 | 26 | | | | | | | | | | | | | | | | | | | | | | |
| | Total Average | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 222 | 288 | 3764 | NA | 215.2 | 74.2 | 18.1 | 418 | 324 | 40 | | | | | | | | | | | | | | | | | | | | | | |
| | Meanmax | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 2: Just The Basics

All these power stats can be complex so we're breaking it down and giving you just the basics

By Allen Lim PhD

Now that we are two stages into the Tour and can actually start looking at averages (albeit only two days worth), I've included the basic data chart that we'll be giving you guys during the race. While I'll be adding other metrics here and there and focusing on different events over the course of the race, this chart will be the basic info that we'll compile each day. It's a lot less detailed than the report that we give the team and riders each night, but there's no way that I could get that much information across online each night, nor would the team be too happy about that. So here are the basics.

In the chart, you'll see a comparison of Floyd vs. the Team. Besides Floyd, the riders on the team who are being monitored include Victor Hugo Pena (The Shark), Nicolas Jalabert (The Poor Ja Ja), Koos Moerenhout (No nickname required), and Robbie Hunter (The Enforcer...for references, see Popovich vs. Hunter at the Tour of Georgia). For both Floyd and the Team, we'll be looking at what happened each day, the average for the Tour and the minimum as well as the maximum values.

Fundamentally, the most important feature is the average power while moving and while pedaling. At this point in time, the guys are trying to get as much "float" time as possible, so there's a fair amount of coasting which brings down the average power when moving while increasing the average power when pedaling.

The next set of data is the work information, which is simply the average power or intensity multiplied by time. I like to break the total work or load into what actually happened vs. what the riders actually felt happened. We're still trying to get our calibration and communication down for the team as a whole so until we nail that down I'm leaving the team info there as "not available."

On really hard days, you'll see big differentials between what happened and what the riders felt happened. Beyond average power and work, it's also important to look at how that power is distributed. For simplicity, I break that into three zones for the riders based on their perception of effort – easy to somewhat hard, somewhat hard to very hard, and anything over very hard. These three zones correlate almost perfectly to time below the lactate threshold, time at lactate threshold, and time above the lactate threshold. From a practical and linguistic standpoint, however, I find it's way easier to just use verbal cues for the zones. The high correlation means it works just the same for describing training distribution.

In the peak power category, the two time frames I find the riders most interested in are their 5-minute power and 30-minute power. The 5-minute power correlates highly with their power at maximal aerobic capacity (i.e., VO2 max), while the 30-minute power correlates highly with their lactate threshold power. Finally, the last feature, I've included is the number of times each rider attacks or surges above 10 watts per kilogram of body weight. Essentially, this is a top end anaerobic effort. It's a bullet and the more bullets a rider fires, the more fatigued they get regardless of the duration or average intensity. So it's a great way to look at how many punches get thrown each day. This is where Floyd suffered a lot last year. Our goal has been not to see that problem this year.

So there's the data for the day. As for other news, everything is pretty steady here at the Phonak camp. Having just left our first hotel, we're all just getting settled into a daily routine - working out the kinks and trying not to rip it to hard yet.

| | | | | | | | | | | |
|-----------------------------|-----------------------|--|---------------------|------|--------------------------------|---------------------------|---------------------------|----------------|--------|---------------------------|
| Name: <u>Michael Landis</u> | | Stage: <u>25, Olpe to Fribourg, 228.7 km</u> | | | | | | | | |
| Stage Place: <u>10th</u> | Time: <u>5:56:11</u> | Date: <u>Sunday, July 2nd</u> | | | | | | | | |
| GC Place: <u>11th</u> | Time: <u>24:10:20</u> | | | | | | | | | |
| | Average Power (W) | | Work (Kjoules) From | | Time (Min) Distributed Between | | | Peak Power (W) | | # of Attacks > 10 W/Kg |
| | Minimum | Maximum | Power | End | Easy to Knee Pain | Knee Hard No Very Hard | Greater Than Very Hard | 5 min | 30 min | |
| | min | max | | | | | | | | |
| Floyd | 195 | 258 | 1534 | 4382 | 222.8 | 68.9 | 37.1 | 922 | 729 | 27 |
| | 201 | 241 | 1529 | 1620 | 203.7 | 59.7 | 31.3 | 399 | 291 | 30 |
| | 195 | 255 | 2072 | 3117 | 173.9 | 89.1 | 25.1 | 549 | 357 | 17 |
| | 193 | 256 | 1934 | 2167 | 222.8 | 68.9 | 37.1 | 922 | 729 | 27 |
| Team | 194 | 261 | 1894 | NA | 212 | 70.2 | 31.8 | 156 | 198 | 21 |
| | 199 | 260 | 1571 | NA | 213 | 61.8 | 32.5 | 178 | 201 | 19 |
| | 196 | 243 | 2293 | NA | 179.2 | 81.8 | 31.1 | 727 | 257 | 16 |
| | 194 | 273 | 1934 | NA | 212.2 | 71.2 | 31.1 | 929 | 729 | 20 |

Prepared by ©Allen Lim

Bicycling

Powerfeed Stage 4: Keeping Floyd Cool

Subtle assistance by fellow teammates is critical to Floyd's success

By Allen Lim PhD

Today was a lot easier for the whole team. With a little bit of rain this morning, it wasn't as hot as it was yesterday and the terrain was less aggressive with only two categorized climbs as opposed to yesterday's five. As you can see from the summary data, the average power outputs were a lot lower. More importantly, there were fewer spikes in power. At any given power output, a steady profile is always easier than one that undulates a lot. With this in mind, the fact that Floyd cut the number of surges he did above 10 watts per kg of body weight by almost half played a key role in a more relaxed ride today.

While the terrain and the steady chase by Quick Step and Davitamon was a major factor contributing to this steadier profile, we noticed that in the previous days downloads that Robbie Hunter's power profiles were significantly steadier than any of his other teammates including Floyd. Floyd joked this morning that it was because no one screws with Robbie. So the suggestion was made that Floyd just follow Robbie. Looks like it helped.

It's this subtle assistance and the less subtle assistance of fetching water bottles or helping Floyd chase back on after a flat that will be critical to Floyd's success here at the Tour. In this first week, the only thing that the team is concerned about is making sure that Floyd has as easy as a ride as possible. In yesterday's race, for example, the guys estimate that they went back to the car close to 10 times for bottles. Most of those bottles ended up going to Floyd as he claims that he drank close to 20 over the stage. Based on the fact that he lost less than 300 grams of total water weight this might have been possible. In contrast, the rest of the team lost between 2 to 3 kilograms of water weight. It's like a bunch of worker bees flapping their wings to keep the queen cool. However it works. One thing is for sure: Floyd is staying pretty hydrated out there.

In the final sprint today, the team also did a fantastic job of bringing Floyd up to the front to keep him out of trouble. Robbie took a monster pull between the 5 km to go and 3 km to go markers and was the only one on the team to lose time. But it was worth it. We're just glad that the crash in the final didn't take Floyd out. Outside of that, it was another pretty uneventful day for us. The real news is that France won the football match tonight. The fireworks are definitely going off now. A day late perhaps, but this far away from home, we'll take what we can get.

Name: Floyd Landis

Stage: 1. Huy to Saint Quentins

Stage Place: 73 Time: 4:59:59

Date: Wednesday, July 26, 2006

GC Place: 9 Time: 19:52:59

| | Average Power (W) | | Work (KJoules) From: | | Time (Min) Distributed Between: | | | Best Power (W) | | # of Attacks 10 W/kg |
|--------------|-------------------|-----------|----------------------|------|---------------------------------|--------------------------|------------------------|----------------|--------|-------------------------|
| | Movene | Podillane | Power | Feet | Easy to Fairly Hard | Fairly Hard to Very Hard | Greater Than Very Hard | 5 min | 30 min | |
| | | | | | | | | | | |
| Today | 209 | 256 | 3740 | 3021 | 200 | 67.9 | 31.1 | 320 | 293 | 54 |
| Tour Average | 206 | 258 | 3691 | 2878 | 198.3 | 65.1 | 33.3 | 301 | 291 | 51 |
| Meanman | 195 | 250 | 3073 | 3113 | 175.5 | 49.3 | 25.5 | 309 | 252 | 22 |
| Maximum | 222 | 278 | 3960 | 4187 | 229.8 | 73.4 | 37.7 | 420 | 329 | 42 |

| | | | | | | | | | | |
|--------------|-----|-----|------|----|-------|-------|------|-----|-----|----|
| Today | 201 | 257 | 3631 | NA | 206.1 | 63.11 | 30 | 402 | 272 | 25 |
| Tour Average | 201 | 261 | 3616 | NA | 202.1 | 64.1 | 31.3 | 392 | 277 | 28 |
| Meanman | 186 | 251 | 2923 | NA | 175.5 | 42.8 | 21.3 | 329 | 235 | 16 |
| Maximum | 222 | 284 | 3964 | NA | 255.2 | 74.2 | 38.7 | 434 | 329 | 50 |

©Created by Allen Lim

Bicycling

Powerfeed Stage 5: An American Invasion

Team Phonak has done more than make American Floyd Landis their team leader--they've embraced his culture

By Allen Lim PhD

When we arrived to the hotel this afternoon we were greeted by the team mechanics in the parking lot. They were thrusting their arms high in the sky as we rolled in like they had just won something, screaming, "Thank You America, Thank You America." I just looked at them and smiled, thinking that they were just being goofballs or making fun of us. But when we got out of the car and we all said our hellos there was a real sincerity to their jocularity.

I had slept most of the way during the transfer and was still shaking off the grogginess as the mechanics met us and waved us over to the hotel restaurant for some food and drink. Cyrille, the lead mechanic here was in a super up-beat mood and immediately grabbed me to ask if I knew where we were, invading all of my personal space in the process. I was thinking that I was somewhere between his left armpit and nose, but I just looked at him and shook my head no. And as I shook, he looked me right in the eye and says, "You are in America." "What?" I ask. "AMERICA!" he enthusiastically replies.

Then it suddenly hit me that we were in the city of Caen, just below the D-Day invasion beaches. Utah, Omaha, Gold, Juno and Sword. As it dawned upon me, Cyrille begins expressing his thanks. In broken English and with a heavy French accent, he asks Jesse and I if we can imagine what it was like. He literally asks us why our country even cared. "If I am an American, I think, who is France. Why I die for this country." He motions like he's holding a machine gun and sounds, "ra-ta-tat-tat...ra-ta-tat-tat," then solemnly sighs, "O Mon Dieu." We get lunch and for the next hour, the staff assigned to work the hotel transfer, gathers around to talk. They ask about how my family immigrated to the U.S. I show them some pictures and they smile and laugh as I bring them back home. It was the first time I've heard one of the Spanish mechanics, Modesto, ever speak English, asking Cyrille how to say certain words so he could talk for himself.

For the most part, I've always felt like an outsider with this team. With the majority of my time spent with TIAA-CREF in the States, my agenda with this program has only been about helping Floyd. But as we sat their laughing and sharing, I realized that these guys were making me a part of their team. I also realized that more than any other team on the Pro Tour, this team - the management, staff, and sponsors, has done more than make an American their team leader. They've embraced his culture.

When I first met Cyrille in January he could barely speak a word of English. Since then English has become the official language of the team and in less than 6 months, he's practically fluent compared to my French. Last year, as I broke down Floyd's data at the races, I was essentially ignored. But in the fall they sent their team doc, Denise, to Boulder to learn more about what I did all year and now I spend my evenings writing reports for the staff and riders, meeting with Floyd and John Lelangué, the team's manager each morning to go over the numbers. In 2007, I-Shares, an American company will be taking over as the title sponsor and they've already shifted to other American products like Giro, Oakley, and Nike. At this year's Tour of California and Tour of Georgia the team sent their "A" squad to support Floyd, placing as much priority if not more on these races than many events here in Europe. Even in this year's Tour, the riders are completely committed to protecting Floyd. While he spent almost all of the first week at last year's Tour drifting solo towards the back, he's almost never out of the top 30 this year and is always surrounded by green and yellow. And finally, after introducing the team to keg sized cans of the caffeinated energy drink "Monster," at the Tour of California, Floyd has got the team addicted and we even have planned delivery dates for this sweet nectar here at the Tour.

Though it might be easy to call it another American invasion, compared to the sacrifices made here over sixty-two years ago, any comparison is moot. The metaphor that this race is a war...well compared to the history around us now, I'd say it's more like a water balloon fight. If there's any amount of importance in that or any social responsibility here, I guess it's that it makes people happy.

Today, Floyd barely avoided a handful of sketchy crashes and ended up having another relatively easy day on the bike. That makes us really happy. Attached below is a summary of the day's stats. Notice, that Floyd (today) and on average and the team (on average) threw a total of 31 water balloons thrown over 10 watts per kg by Floyd today (I checked the data...a weird quirk). But at some of the lowest average power outputs, they'll all be ready to fight tomorrow.

Name: Floyd Landis Stage: 5, Bouviers to Caen
 Stage Place: 34 Time: 5:18:50 Date: Thursday, July 26
 GC Place: 8 Time: 1:11:19

| | Average Power (W) | | Work (Kjoules) From | | Time (Min) Distributed Between: | | | Best Power (W) | | # of Attacks |
|--------------|-------------------|-----------|---------------------|------|---------------------------------|----------------------------|---------------------------|----------------|-----------|--------------|
| | Moving | PedalSpin | Power | Feet | Easy to Early Hard | Early Hard to Very Hard | Greater Than Very Hard | 5 min | 10 min | |
| Floyd Landis | | | | | | | | | | |
| Today | 196 | 242 | 3749 | 3970 | 226 | 62 | 30 | 413 | 281 | 31 |
| Team Average | 204 | 255 | 3703 | 3897 | 204.3 | 64.12 | 32.9 | 403 | 289 | 31 |
| Minimum | 185 | 242 | 3075 | 3113 | 175.5 | 49.3 | 25.3 | 369 | 252 | 22 |
| Maximum | 232 | 276 | 3969 | 4187 | 229.8 | 73.4 | 57.7 | 420 | 329 | 42 |
| Team | | | | | | | | | | |
| Today | 106 | 245 | 3750 | NA | 229.4 | 60.5 | 29.03 | 394 | 273 | 31 |
| Team Average | 201 | 257 | 3662 | NA | 208.5 | 61.5 | 30.5 | 390 | 276 | 28 |
| Minimum | 186 | 245 | 2925 | NA | 175.5 | 43.8 | 21.3 | 329 | 235 | 16 |
| Maximum | 232 | 284 | 3941 | NA | 235.2 | 74.2 | 38.1 | 434 | 329 | 50 |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 6: Looking Beyond the Surface

The numbers say the day was difficult, but American Floyd Landis felt otherwise

By Allen Lim PhD

If you just look at the numbers from today, you would think that this was the hardest stage of the Tour to date for Floyd. There were definitely new highs today - a 223-watt moving average (new max), a 275-watt average while pedaling (second highest by 1-watt), and a new maximum best 5-minute power output of 422 watts. But if you watched Floyd and the guys at dinner tonight, you would've thought that they just cruised around the park today. They were talkative, cracking jokes, and really relaxed. When I asked Floyd how the day felt, he commented that it was probably the easiest day so far. "Oh ya, it was easy," he remarked. "It was finally cool. It felt good, I could actually do some work."

Things are rarely what they appear on the surface and sometimes it's important to take a closer look. Today the real story was the weather giving the guys on the team some reprieve from what felt like oppressive heat on stage 3 and 4. It cooled down a lot yesterday as well which gave the guys an additional day to unwind before tomorrow's long time trial. In addition, the normally strong winds around Normandy we're relatively calm over the last two days and played no factor for us on either day of racing. While the riders did have a bit of a headwind in yesterday's stage, it was actually welcome.

As Floyd explains it, "It's always easier sitting in when there's a head wind. The speeds are lower and you get more draft." And he's right. Whenever he's sitting on and drafting into a head wind, his average power outputs are always lower compared to sitting in a tail wind. Today, there was slightly more cross and tail wind sections, and that was reflected by an increase in average power while moving from 196 watts yesterday to 218 watts today for the team. Similarly, Floyd's average from yesterday of 196 watts increased to 223 watts today. This same pattern was observed in the team and Floyd's power output while pedaling. And yet, from the rider's perspective today wasn't as difficult.

The initial heat stress experienced by the riders also resulted in what may initially appear to be surprising increases in body weights. For stage 1, 2, 3, and 4, the average body weights for the team were 71.47, 71.50, 71.49, and 71.58 kilograms before the start of each respective stage. These values aren't just pretty consistent, they're statistically insignificant differences. But on the morning of stage 4, every rider on the team showed an increase in body weight and the team average went up to 72.46 kilograms - an increase of 0.875 kilograms for the team. So what gives? Well, if we rate the heat index or load the riders faced on each stage, or even just look at the bottle count across the stages, we'd see that the heat load faced by the riders was normal, hot, way too hot, normal, cool, and almost cold for stage 1 through 6, respectively. That sudden increase in body weight occurred on stage 5, three days after the first really hot day at this year's Tour and after two continuous days of hot conditions.

Based on the timing, this increase is a normal part of heat acclimatization. While it may take a person who isn't use to exercising in the heat up to 2 weeks to adapt, elite athletes and individuals who have been previously exposed to exercise in hot weather can respond much more rapidly. For our population of riders, one of the first adaptations to the heat is an increase in plasma volume (i.e., an increase in plasma or fluid in blood) and drops in resting and exercising heart rates due to a net increase in the total cardiac output. This type of plasma volume expansion can range from 5 to 30%, and assuming a blood volume of 5 liters, may result in an increase in fluid weight of .25 to 1.5 kilograms. So we're in the ballpark. Normally, this expansion is also accompanied by an increase in salt retention and when the doctor checked the rider's electrolyte levels yesterday, there was a significant increase in sodium. And of course, because blood is composed of plasma and red blood cells, any increase in plasma would dilute red blood cell concentration. While this might scare many athletes, the fact of the matter is that it's not an athlete's hematocrit or concentration of red blood cells that matters, it's their total red blood cell mass. Keeping the same mass and increasing plasma which dilutes that mass actually improves performance as the output of the heart actually increases.

Ultimately, looking beyond the surface is often revealing. Power, weather, and an athlete's perception - these are all factors that play into performance. As we prepare for tomorrow's long time trial, reviewing and checking all of the elements of a fast ride is at forefront of all of our minds. So before I leave for the night, I'll leave you with one last thought. Amongst professional cyclists who are already so similar in their ability to produce power, it's unlikely that the strongest rider will win tomorrow's time trial. The winner will be one of the most aerodynamic. And with Floyd's seemingly "funny" position, we're feeling pretty good.

Name: Floyd Landis

Stage: 6, L'Etape de France

Stage Place: 59 Time: 4:10:17
GC Place: 8 Time: 29:21:36

Date: Friday, July 7th

| | | | | | | Time (Min) Distributed Between: | | | Best Power (W) | | # of Attacks |
|--------------|--------------|-------------------|-----------|----------------------|------|---------------------------------|--------------|--------------|----------------|--------|--------------|
| | | Average Power (W) | | Work (Kjoules) From: | | Easy to | Fairly Hard | Greater Than | 5 min | 30 min | Greater Than |
| | | Moving | Pedalling | Power | Feet | Fairly Hard | to Very Hard | Very Hard | | | 10 W/Kg |
| Floyd Landis | Today | 223 | 275 | 3349 | 3415 | 157 | 58 | 35 | 422 | 283 | 25 |
| | Tour Average | 207 | 258 | 3653 | 3529 | 197.6 | 63.54 | 32.69 | 407 | 258 | 10 |
| | Minimum | 195 | 242 | 3075 | 3123 | 157 | 49.3 | 25.2 | 369 | 252 | 22 |
| | Maximum | 223 | 276 | 3969 | 4187 | 229.8 | 73.4 | 37.7 | 422 | 329 | 42 |
| Team | Today | 218 | 279 | 3280 | NA | 160.4 | 57.1 | 32.8 | 427 | 281 | 26 |
| | Tour Average | 194 | 249 | 3444 | NA | 192 | 60.3 | 29.7 | 398 | 280 | 28 |
| | Minimum | 186 | 239 | 2925 | NA | 157.3 | 32.9 | 21.3 | 329 | 235 | 16 |
| | Maximum | 231 | 292 | 3934 | NA | 235.2 | 74.2 | 38.1 | 446 | 329 | 40 |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 7: Everything Counts

Bad luck impacts Landis' time

By Allen Lim PhD

Back in early January, I was sitting in Floyd's garage as he was showing off his latest time trial innovations. He had recently sawed apart some black PVC pipe and hand bonded two sliced halves together to create armrests that literally cuffed his wrists together. They were zip tied near the top of his aero-bars as were a similar but wider (bigger pipe) set of pads closer to the bottom for his elbows. He was pretty excited about his latest creation. As he got on the bike to demonstrate the new position, I got pretty excited too. I imagine that if we worked some duck tape into the party we would've really impressed the ladies.

A month later, we reconvened at the Allied Aerospace wind tunnel in San Diego with a cast of characters that included Scott "the human wind tunnel" Warren, Mark Lucas a coach on scholarship from New Zealand, and members of the Phonak squad who brought a box full of goodies from BMC. After getting reference values for Floyd's 2005 position and equipment, we spent the next few days methodically testing part after part, body position after body position. We even broke out the clay, cardboard, and, of course, the duck tape. But the biggest revelation was Floyd's garage tinkered bars. After reconfirming the results and smiling as Floyd got his "I told you so" in with the team who never really felt his position was quite right, I thought to myself, "this guy knows speed."

Although it's initially difficult to see all of the changes made in Floyd's equipment and position from 2005 to 2006, the list of modifications is extensive. They include changes to his arm extensions, arm pads, handlebar fairing, water bottle, crank, stem, seat post, skewers, helmet, arm angle, arm width, seat position, and elbow placement. Each of these changes removed grams of drag -- in some instances 15 to 20 grams and in other instances just over a 100. While this might seem a bit obsessive, even minute changes in aerodynamic drag can result in enormous changes in power.

Specifically, aerodynamic drag changes exponentially with speed, while the power to overcome aerodynamic drag changes cubically with speed. That means if you want to double your speed on a flat road, you need to increase your power eight-fold. Another way to think about it is to realize that at 30 mph an average professional cyclist may only have about 2600 grams or 2.6 kilograms of drag holding them back. You could literally lasso a cyclist galloping along at 30 mph and pull back with a force equivalent to dropping a 6-pound weight and stop them in their tracks. Most kids can curl more than that. Moreover, relative to the weight bike manufacturers attempt to shave off their frames to help overcome gravitational resistance, the amount of speed gained going uphill by dropping a pound of weight off your bike is negligible compared to a pound of drag on the flats.

In today's time trial, some bad luck and the subsequent addition of some extra grams of drag may not have lost him the race, but these events definitely had an impact on his final time. Before the start, we modeled that in the worst-case scenario, Floyd would finish with a time of 1 hour, 2 minutes, and 6 seconds. In the best-case scenario he would finish in a time of 1 hour, 1 minute, and 35 seconds. This 31-second spread amounts to a power range of only 10-watts. In reality, Floyd came in at 1 hour, 2 minutes, and 44 seconds for 2nd place - a whole 38 seconds off our worst-case scenario, 70 seconds off of what we hoped, and 60 seconds off the winning time. So what happened?

Well, the first thing that happened was Floyd had a bit of a mishap. At kilometer 13 he hit a speed bump and this bar extensions snapped clean off. It was amazing he didn't crash. So Floyd gets a bike change, losing valuable time, and switches to a spare bike that is missing the new prototype water bottle that was on his first. Then, and I can't even believe this happened, but his bars on the second bike slipped forward. After reviewing some of our wind tunnel data, I estimate that the loss of the bottle and the change in handlebar position, gave Floyd 80-grams of additional drag. At his velocity and assuming the length of 52 kilometers this is equivalent to an additional 38-seconds. So had Floyd been on his original bicycle, he would only be about 23-seconds out of first place. And that 23-seconds could've easily been lost in the bike change. On T.V., it looked like the time lost was about 20 seconds. Assuming a 20 second bike change and 80-grams of extra drag, Floyd still might have lost by about 3 seconds or a 1/4 to 1/2 of a watt. In any case, that's bike racing. Everything counts but in the end, it is what it is. Things should've, could've and would've been better if this and if that. But who really knows. We're all just glad that he's safe and still had a great ride despite a few problems. He kept it cool today and based on his "virtual" power that I've modeled between 410 to 417 watts he's on track for a great Tour.

| | Time | | | Power |
|--|------|-----|-----|-------|
| | Hrs | Min | Sec | |
| Floyd's actual time plus 80 grams of extra drag: | 1 | 2 | 44 | 410 |
| Floyd's actual time with a 20 sec bike change and 80 grams of extra drag: | 1 | 2 | 44 | 417 |
| Floyd's Virtual Time without 80 extra grams of drag: | 1 | 2 | 6 | 410 |
| Floyd's virtual time without a 20 second bike change and without 80 extra grams of drag: | 1 | 1 | 46 | 417 |

Prepared by ©Allen Lim

Bicycling

Powerfeed Stage 8: Bridging The Gap

The gap between the next generation of American cyclists and Floyd boils down to time and hard work.

By Allen Lim PhD

Note:

Because of the rest day tomorrow and the transfer down to Bordeaux, the bicycles won't be arriving until late tonight and the riders won't be here until tomorrow. As a result, I'll be posting the power data from Floyd and the team for today's stage tomorrow after I catch up with them. From what I've been told, the team had to do some work today to keep the breakaway in check, but neither the team nor Floyd were all that stressed.

Since we weren't going to get the computers this evening I thought I'd review some data from Floyd's Tour of Georgia to write about. But that got me thinking about other things, so on the car drive down, I wrote the following story. And yes, I am really carsick now.

Bridging the Gap:

It had been a long day in the car and I was anxious to get back to the hotel. I was following Mike Friedman, Brad Huff, Rahsaan Bahati, and Mike Creed - members of Team TIAA-CREF and some of America's most talented up and coming cyclists - on a training ride in the Pinos Altos National Park just outside of Silver City New Mexico. It was the first week of February and most of us had already been on the road for the past two months. Road weary does not even come close to describing how we all felt. The 5 hours in the car that day felt like an eternity and I was beat. But it was a small thing compared to the weariness I could see in the boys. We still had 45 minutes to go, but every mile the speed steadily waned. So when I saw Rahsaan pull off the road exhausted, my first instinct was to just pack him up in the car. His day was over. Or so it appeared.

As I pulled over to talk to Rahsaan, his head hung low over his handlebars and he straddled his bike like a rag doll. I got out of the car and walked over but he didn't look like he wanted to talk to anyone. I approached slowly to give him some time and to figure out what I was going to say. But before I reached him, Mike Creed who had been slaughtering the guys all day rolled over and crouched down so that his head was even with Rahsaan's.

Angry, surly, and intense are just a few adjectives to describe Creed and I felt a twinge of apprehension as I prepared to listen to what he was about to levy on Rahsaan. But the words that came out of Creed's mouth weren't mean or aggressive. They were gentle and calm. "I know it hurts man. I know you want to stop," he said, "but this is the only way. This is the only way." Before I could say anything, Rahsaan took a deep breath, clipped back into his pedals and got back on the road. And as I got back in the car to follow, those simple words kept echoing in my head. "This is the only way...this is the only way."

What will it take to bring the next generation of American's to the Tour and are we even capable? As I sit here in France watching Floyd take on the World, my mind keeps drifting back to that day outside of Silver City and the enormity of what lies ahead for those young men. But what I've come to realize, what I believe, is that the U.S. is home to some of the most talented cyclists in the world - that when it comes to raw power, we could fill the peleton here at the Tour with a pretty sizable cottage of wattage. But power and talent don't always mean results and at the end of the day there is no prize for best 5-minute power.

This point hit home this morning when I was reviewing Floyd's power data from the Brasstown Bald stage at this year's Tour of Georgia and decided to compare his numbers and results with the power data I gathered from TIAA-CREF rider Will Frishkorn. On this particular day, Floyd redeemed himself with an amazing ride up Brasstown Bald, holding onto the wheel of climbing phenom Tom Danielson to ensure his overall win at Georgia a year after losing to Tom by seconds. Seventy some odd riders and over 18 minutes later, Will rolled across the line. That's not just a small gap, that's a whole other race.

But when you look at the numbers for Floyd and Will you wouldn't be able to predict the chasm in the results. As an example, Will finished with a higher average power output while moving and while pedaling, did more work, spent more time between 4 to 6 watts per kg and above 6 watts per kg, and had a higher peak power output for 5-minutes than Floyd. In addition, he was about a kilogram lighter than Floyd at the time, which significantly raises his power to weight ratio.

So what happened? Well basically, Will had an incredible ride, but his goals that day were different from Floyd's. Will's job was to help Lucas Euser, our team's KOM leader into an early breakaway. Not only did Will do this, he drove the break taking two pulls for each pull taken by the 4 other breakaway companions with Lucas sitting on. After helping Lucas make it to the KOM climbs so he could duke it out with Jason McCartney, he throttled back and rode in easy over Unicoi Gap and Brasstown Bald. As a side note, we saw the same higher power values in the Phonak riders who spent all day working to support Floyd.

The fact that race tactics plays such an important role in both an athlete's power profile as well as the end result is one of the reason's why Floyd is so open about sharing his power information. Beyond tactics, terrain, weather, food intake, and mindset are also critical determinants of performance. Thus, you can't assume you know anything about the rider or the race by just looking at the numbers. As a result, Floyd knows he's really not giving anything away unless there's another Floyd Landis in the peleton doing the same ride and having the same experiences. At the end of the day, he is the only one who understands the myriad of factors that impact his race.

With that in mind, the numbers are extremely telling if you don't ignore and stay aware of how these factors impact you. In fact, having the power data is the best tool we have in parsing out all of these complex variables. It helps us to understand if the differences we see between riders are actually physiologic or psychological, technical or cultural, giving us references that are critical to goal setting.

So if we discover a cyclist who is only a 1/2 watt per kg away from being capable of winning the Tour, we can set goals to help that athlete develop. But it's not just, hey you need to do an extra 1/2 watt per kg, it's you need to do an extra 1/2 watt per kg when you don't want to be at the races, when it's cold and miserable, when you're missing your girlfriend, don't speak the language, are missing your bags, and feel terrible. Because the bottom line as a pro is it's your job now. And sometimes, going to work sucks. In the end the gap between the next generation of American cyclists and Floyd boils down to time and hard work. It's been and will continue to be the only way.

Three months after Rahsaan had his rear handed to him on that training ride outside of Silver City, he returned for the Gila Stage race. On one of the stages the team rode brilliantly to bring back a breakaway and lead him out for the sprint, but he didn't come through for the guys. The next day, however, in the criterium he made us all proud when he lit up the sprint for an impressive win. It was the first time an African American has ever won a race on the US National Racing Calendar. Perhaps in a few years down the Champs Elysees, history will be made again.

| Unicoi Gap | | | | | | |
|------------|------|------|-----|-------|------|---------|
| | Min | Sec | Km | Power | W/Kg | Cadence |
| Floyd | 13.0 | 38.0 | 5.5 | 376.0 | 5.4 | 90.0 |
| Will | 16.0 | 58.0 | 5.5 | 318.0 | 4.6 | 77.0 |

| Brasstown | | | | | | |
|-----------|------|------|-----|-------|------|---------|
| | Min | Sec | Km | Power | W/Kg | Cadence |
| Floyd | 23.0 | 28.0 | 7.0 | 402.0 | 5.7 | 82.0 |
| Will | 32.0 | 12.0 | 7.0 | 298.0 | 4.3 | 65.0 |

Created by ©Allen Lim

Tour of Georgia: Brasstown Bald Stage

| | Peak Power (Watts) | | Peak Power (Watts per Kg) | |
|-------------|--------------------|-----------|---------------------------|-----------|
| | 5-minute | 10-minute | 5-minute | 10-minute |
| Floyd (2nd) | 440 | 398 | 6.29 | 5.69 |
| Will (79th) | 443 | 346 | 6.42 | 5.01 |

Created by ©Allen Lim

Tour of Georgia: Brasstown Bald Stage

| | Moving | Pedaling | Wash | Time (Min) Distributed Between a W/Kg | | |
|-------------|--------------|--------------|--------------|---------------------------------------|--------|------|
| | Watts - W/Kg | Watts - W/Kg | Watts - W/Kg | > 4 | 4 to 6 | > 6 |
| Floyd (2nd) | 372 | 388 | 118 | 3.58 | 4.104 | 4.17 |
| Will (79th) | 292 | 4.33 | 332 | 4.68 | 4.68 | 4.68 |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 9: Floyd's Avascular Necrosis

Floyd is human, he's vulnerable, and he feels pain - everyday

By Allen Lim PhD

I wasn't quite sure how to approach today's story, so I went and asked Floyd what he thought I should say. His response was simple. "Just talk about it from your perspective," he told me.

My perspective? I can't even begin to imagine that my perspective can do the situation justice. And I guess from my perspective we've kept it a secret for so long that I don't even know how to talk about it without wanting to hide from people the misery I've seen him go through. Because it means he's human, he's vulnerable, and he feels pain - everyday.

I found out that Floyd had avascular necrosis the very first day I met him. We were supposed to be talking about his upcoming camp, but after a little bit of small talk, he just started pebbling me with questions. "What do you know about avascular necrosis?" "Do you think I can ride with an artificial hip?" "How long do you think I can go before I need to get it a new one?" I was completely unprepared.

About a month later, Floyd had surgery to try and increase the blood flow to his right hip. A hip that from every x-ray and MRI I saw was just thrashed. Bone on bone - an oddly shaped and atrophied shaft that fit into the socket like a square peg rammed into a circle. He didn't tell me about the surgery until after he had it done and when I asked if he told the team, he told me no. I understood. It had been a rough enough transition from the U.S. Postal Cycling Team to Phonak and he was afraid he'd lose his job. So from that point on, we stopped talking about his hip. Instead it was his finger.

I could always see the misery in his face when it hurt. Simple things like walking, getting out of a chair, and straddling his bike before a ride were difficult. If you didn't know him, you'd mistake the look for anger or a sarcastic scowl. His training and racing were at times erratic and he generally opted to ride alone so that he could hide his obvious discomfort from others. Sadly, I often felt that the energy it took to hide it wore on him more than the grinding mass inside him.

When we talked tonight he was the most relaxed I think I've ever seen him. It was as if telling the world had lifted an enormous weight. We joked that it was one less thing he had to worry about now, and one more thing for everyone else to think about. That he was giving everyone the "finger." But then he asked, "Do you think I'll be here next year?" I said, "You'll be here next year." He paused and whispered, "I'm scared." I had no reply.

All I know is that he needed to tell people what was really going on and that he couldn't wait. He had to free himself of the burden before tomorrow's stage when the Tour really begins. He didn't want to make excuses, but he was sick of people assuming that they knew what was really going on.

I asked him, "Do you remember the tunnel." He laughed and said, "Ha! They must of thought I was a real jerk." I rolled my eyes and said, "Ya you were a real ass." We went to the tunnel because we needed to find a position that wasn't just more aerodynamic, but one he could pedal in. He built those bars because it was the only way he could open up his hip angle. But nobody else knew that. And when they suggested a different position, one that appeared even faster than his position now, he just walked out and curtly told everyone, "I'm done."

I sat there not saying a word as the others shook their heads in dismay and disgust. I sat there and thought about the first week of last year's Tour. About how he'd sometimes come into the bus after a stage and tell me how he thought he was going to vomit, the pain was so bad. But it never stopped him.

We all have fears. After all, the world is an uncertain place. But when you accept it, when you bathe in it for a long enough time, the surprising thing is that it becomes all right. You can't hide from it cause it'll find you. As long as you keep on moving forward...as long as you don't give up there will always be enough hope to balance the doubt. So we're going to keep moving forward. One day at a time. Cause as Floyd puts it, "I'm 100% of what I am now." And he's right. That's just who he is - one hundred percent all the time. Kind of puts all the data in a different perspective doesn't it.

| Team: USA Cycling | | Stage: 9: Powerfeed Stage 9 | |
|-------------------|---------|-----------------------------|---------|
| Rank | Time | Rank | Time |
| 1 | 1:00:00 | 1 | 1:00:00 |
| 2 | 1:00:00 | 2 | 1:00:00 |
| 3 | 1:00:00 | 3 | 1:00:00 |
| 4 | 1:00:00 | 4 | 1:00:00 |
| 5 | 1:00:00 | 5 | 1:00:00 |
| 6 | 1:00:00 | 6 | 1:00:00 |
| 7 | 1:00:00 | 7 | 1:00:00 |
| 8 | 1:00:00 | 8 | 1:00:00 |
| 9 | 1:00:00 | 9 | 1:00:00 |
| 10 | 1:00:00 | 10 | 1:00:00 |
| 11 | 1:00:00 | 11 | 1:00:00 |
| 12 | 1:00:00 | 12 | 1:00:00 |
| 13 | 1:00:00 | 13 | 1:00:00 |
| 14 | 1:00:00 | 14 | 1:00:00 |
| 15 | 1:00:00 | 15 | 1:00:00 |
| 16 | 1:00:00 | 16 | 1:00:00 |
| 17 | 1:00:00 | 17 | 1:00:00 |
| 18 | 1:00:00 | 18 | 1:00:00 |
| 19 | 1:00:00 | 19 | 1:00:00 |
| 20 | 1:00:00 | 20 | 1:00:00 |
| 21 | 1:00:00 | 21 | 1:00:00 |
| 22 | 1:00:00 | 22 | 1:00:00 |
| 23 | 1:00:00 | 23 | 1:00:00 |
| 24 | 1:00:00 | 24 | 1:00:00 |
| 25 | 1:00:00 | 25 | 1:00:00 |
| 26 | 1:00:00 | 26 | 1:00:00 |
| 27 | 1:00:00 | 27 | 1:00:00 |
| 28 | 1:00:00 | 28 | 1:00:00 |
| 29 | 1:00:00 | 29 | 1:00:00 |
| 30 | 1:00:00 | 30 | 1:00:00 |
| 31 | 1:00:00 | 31 | 1:00:00 |
| 32 | 1:00:00 | 32 | 1:00:00 |
| 33 | 1:00:00 | 33 | 1:00:00 |
| 34 | 1:00:00 | 34 | 1:00:00 |
| 35 | 1:00:00 | 35 | 1:00:00 |
| 36 | 1:00:00 | 36 | 1:00:00 |
| 37 | 1:00:00 | 37 | 1:00:00 |
| 38 | 1:00:00 | 38 | 1:00:00 |
| 39 | 1:00:00 | 39 | 1:00:00 |
| 40 | 1:00:00 | 40 | 1:00:00 |
| 41 | 1:00:00 | 41 | 1:00:00 |
| 42 | 1:00:00 | 42 | 1:00:00 |
| 43 | 1:00:00 | 43 | 1:00:00 |
| 44 | 1:00:00 | 44 | 1:00:00 |
| 45 | 1:00:00 | 45 | 1:00:00 |
| 46 | 1:00:00 | 46 | 1:00:00 |
| 47 | 1:00:00 | 47 | 1:00:00 |
| 48 | 1:00:00 | 48 | 1:00:00 |
| 49 | 1:00:00 | 49 | 1:00:00 |
| 50 | 1:00:00 | 50 | 1:00:00 |
| 51 | 1:00:00 | 51 | 1:00:00 |
| 52 | 1:00:00 | 52 | 1:00:00 |
| 53 | 1:00:00 | 53 | 1:00:00 |
| 54 | 1:00:00 | 54 | 1:00:00 |
| 55 | 1:00:00 | 55 | 1:00:00 |
| 56 | 1:00:00 | 56 | 1:00:00 |
| 57 | 1:00:00 | 57 | 1:00:00 |
| 58 | 1:00:00 | 58 | 1:00:00 |
| 59 | 1:00:00 | 59 | 1:00:00 |
| 60 | 1:00:00 | 60 | 1:00:00 |
| 61 | 1:00:00 | 61 | 1:00:00 |
| 62 | 1:00:00 | 62 | 1:00:00 |
| 63 | 1:00:00 | 63 | 1:00:00 |
| 64 | 1:00:00 | 64 | 1:00:00 |
| 65 | 1:00:00 | 65 | 1:00:00 |
| 66 | 1:00:00 | 66 | 1:00:00 |
| 67 | 1:00:00 | 67 | 1:00:00 |
| 68 | 1:00:00 | 68 | 1:00:00 |
| 69 | 1:00:00 | 69 | 1:00:00 |
| 70 | 1:00:00 | 70 | 1:00:00 |
| 71 | 1:00:00 | 71 | 1:00:00 |
| 72 | 1:00:00 | 72 | 1:00:00 |
| 73 | 1:00:00 | 73 | 1:00:00 |
| 74 | 1:00:00 | 74 | 1:00:00 |
| 75 | 1:00:00 | 75 | 1:00:00 |
| 76 | 1:00:00 | 76 | 1:00:00 |
| 77 | 1:00:00 | 77 | 1:00:00 |
| 78 | 1:00:00 | 78 | 1:00:00 |
| 79 | 1:00:00 | 79 | 1:00:00 |
| 80 | 1:00:00 | 80 | 1:00:00 |
| 81 | 1:00:00 | 81 | 1:00:00 |
| 82 | 1:00:00 | 82 | 1:00:00 |
| 83 | 1:00:00 | 83 | 1:00:00 |
| 84 | 1:00:00 | 84 | 1:00:00 |
| 85 | 1:00:00 | 85 | 1:00:00 |
| 86 | 1:00:00 | 86 | 1:00:00 |
| 87 | 1:00:00 | 87 | 1:00:00 |
| 88 | 1:00:00 | 88 | 1:00:00 |
| 89 | 1:00:00 | 89 | 1:00:00 |
| 90 | 1:00:00 | 90 | 1:00:00 |
| 91 | 1:00:00 | 91 | 1:00:00 |
| 92 | 1:00:00 | 92 | 1:00:00 |
| 93 | 1:00:00 | 93 | 1:00:00 |
| 94 | 1:00:00 | 94 | 1:00:00 |
| 95 | 1:00:00 | 95 | 1:00:00 |
| 96 | 1:00:00 | 96 | 1:00:00 |
| 97 | 1:00:00 | 97 | 1:00:00 |
| 98 | 1:00:00 | 98 | 1:00:00 |
| 99 | 1:00:00 | 99 | 1:00:00 |
| 100 | 1:00:00 | 100 | 1:00:00 |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 10: The Mountains

It's nice to get this show going and boy is it going

By Allen Lim PhD

We are finally in the mountains. It's nice to get this show going and boy is it going. Today, Floyd hit new maximums for average power, total work, time spent at and above his lactate threshold, peak power for 5 minutes, and peak power for 30 minutes. And yet, the day still wasn't quite as hard as it could have been.

The first major climb today was the Col d'Osquich. It started 44 km into the race and took 14 minutes for an average power output of 344 watts. That wattage is actually lower than the average 10 minute best that Floyd has already accumulated through this race, so it was pretty light. The next climb of the day was the Col de Soudet, 87 km into the race. That took the front group 44 minutes to climb and Floyd averaged 355 watts up it. Again, well within limits. And finally, the Col de Marie Blanche, 141 km into the race took the main peloton 28 minutes for an average of 366 watts for Floyd.

Still, many riders including some from our squad did have some difficulty. The transition from riding the flats to the climbs can be pretty rough so the team's staying pretty relaxed about the situation. Part of the difficulty probably stemmed from the fact that there were a lot more riders around fighting for position at the base of those three climbs. In Floyd's power profile, the surges leading up to those climbs were frequent and volatile. Though, he didn't surge much above 10 watts per kg he had almost 500 spikes above 6 watts per kg. Although those surges only last for 1 to 10 seconds on average, there enough to tenderize even the toughest rider after a while.

Otherwise, there really aren't too many dramatic stories to tell about the day. I bumped into Dave Zabriskie and he sang for me at the start, Tom Boonen showed me all of the different World Championship decals on his bicycle, Christian VandeVelde waxed philosophic about a big chocolate croissant he had for breakfast, and Floyd recited a bunch of Jack Handy quotes. One about seeing someone fall on an ant and not laughing cause you could've been that ant was particularly funny. Finally, when I got to the hotel today, the staff and I had a really good time watching the race on T.V. At fist we were all screaming and yelling, but after about an hour all 5 of us fell asleep. That was a good nap. So that's about it for today. The race has begun. Tomorrow won't be as easy. But I think I'll still take a nap.

| Powerfeed Stage 10: The Mountains | | | | | | | | | |
|-----------------------------------|------|-------|------|------|----------------------------|------|-------|------|------|
| Stage 10: The Mountains | | | | | Total 10 Stages: 10 Stages | | | | |
| Stage | Time | Power | Work | Time | Stage | Time | Power | Work | Time |
| 1 | 1:00 | 100 | 1000 | 1:00 | 10 | 1:00 | 100 | 1000 | 1:00 |
| 2 | 1:00 | 100 | 1000 | 1:00 | 11 | 1:00 | 100 | 1000 | 1:00 |
| 3 | 1:00 | 100 | 1000 | 1:00 | 12 | 1:00 | 100 | 1000 | 1:00 |
| 4 | 1:00 | 100 | 1000 | 1:00 | 13 | 1:00 | 100 | 1000 | 1:00 |
| 5 | 1:00 | 100 | 1000 | 1:00 | 14 | 1:00 | 100 | 1000 | 1:00 |
| 6 | 1:00 | 100 | 1000 | 1:00 | 15 | 1:00 | 100 | 1000 | 1:00 |
| 7 | 1:00 | 100 | 1000 | 1:00 | 16 | 1:00 | 100 | 1000 | 1:00 |
| 8 | 1:00 | 100 | 1000 | 1:00 | 17 | 1:00 | 100 | 1000 | 1:00 |
| 9 | 1:00 | 100 | 1000 | 1:00 | 18 | 1:00 | 100 | 1000 | 1:00 |
| 10 | 1:00 | 100 | 1000 | 1:00 | 19 | 1:00 | 100 | 1000 | 1:00 |
| 11 | 1:00 | 100 | 1000 | 1:00 | 20 | 1:00 | 100 | 1000 | 1:00 |
| 12 | 1:00 | 100 | 1000 | 1:00 | 21 | 1:00 | 100 | 1000 | 1:00 |
| 13 | 1:00 | 100 | 1000 | 1:00 | 22 | 1:00 | 100 | 1000 | 1:00 |
| 14 | 1:00 | 100 | 1000 | 1:00 | 23 | 1:00 | 100 | 1000 | 1:00 |
| 15 | 1:00 | 100 | 1000 | 1:00 | 24 | 1:00 | 100 | 1000 | 1:00 |
| 16 | 1:00 | 100 | 1000 | 1:00 | 25 | 1:00 | 100 | 1000 | 1:00 |
| 17 | 1:00 | 100 | 1000 | 1:00 | 26 | 1:00 | 100 | 1000 | 1:00 |
| 18 | 1:00 | 100 | 1000 | 1:00 | 27 | 1:00 | 100 | 1000 | 1:00 |
| 19 | 1:00 | 100 | 1000 | 1:00 | 28 | 1:00 | 100 | 1000 | 1:00 |
| 20 | 1:00 | 100 | 1000 | 1:00 | 29 | 1:00 | 100 | 1000 | 1:00 |
| 21 | 1:00 | 100 | 1000 | 1:00 | 30 | 1:00 | 100 | 1000 | 1:00 |
| 22 | 1:00 | 100 | 1000 | 1:00 | 31 | 1:00 | 100 | 1000 | 1:00 |
| 23 | 1:00 | 100 | 1000 | 1:00 | 32 | 1:00 | 100 | 1000 | 1:00 |
| 24 | 1:00 | 100 | 1000 | 1:00 | 33 | 1:00 | 100 | 1000 | 1:00 |
| 25 | 1:00 | 100 | 1000 | 1:00 | 34 | 1:00 | 100 | 1000 | 1:00 |
| 26 | 1:00 | 100 | 1000 | 1:00 | 35 | 1:00 | 100 | 1000 | 1:00 |
| 27 | 1:00 | 100 | 1000 | 1:00 | 36 | 1:00 | 100 | 1000 | 1:00 |
| 28 | 1:00 | 100 | 1000 | 1:00 | 37 | 1:00 | 100 | 1000 | 1:00 |
| 29 | 1:00 | 100 | 1000 | 1:00 | 38 | 1:00 | 100 | 1000 | 1:00 |
| 30 | 1:00 | 100 | 1000 | 1:00 | 39 | 1:00 | 100 | 1000 | 1:00 |
| 31 | 1:00 | 100 | 1000 | 1:00 | 40 | 1:00 | 100 | 1000 | 1:00 |
| 32 | 1:00 | 100 | 1000 | 1:00 | 41 | 1:00 | 100 | 1000 | 1:00 |
| 33 | 1:00 | 100 | 1000 | 1:00 | 42 | 1:00 | 100 | 1000 | 1:00 |
| 34 | 1:00 | 100 | 1000 | 1:00 | 43 | 1:00 | 100 | 1000 | 1:00 |
| 35 | 1:00 | 100 | 1000 | 1:00 | 44 | 1:00 | 100 | 1000 | 1:00 |
| 36 | 1:00 | 100 | 1000 | 1:00 | 45 | 1:00 | 100 | 1000 | 1:00 |
| 37 | 1:00 | 100 | 1000 | 1:00 | 46 | 1:00 | 100 | 1000 | 1:00 |
| 38 | 1:00 | 100 | 1000 | 1:00 | 47 | 1:00 | 100 | 1000 | 1:00 |
| 39 | 1:00 | 100 | 1000 | 1:00 | 48 | 1:00 | 100 | 1000 | 1:00 |
| 40 | 1:00 | 100 | 1000 | 1:00 | 49 | 1:00 | 100 | 1000 | 1:00 |
| 41 | 1:00 | 100 | 1000 | 1:00 | 50 | 1:00 | 100 | 1000 | 1:00 |
| 42 | 1:00 | 100 | 1000 | 1:00 | 51 | 1:00 | 100 | 1000 | 1:00 |
| 43 | 1:00 | 100 | 1000 | 1:00 | 52 | 1:00 | 100 | 1000 | 1:00 |
| 44 | 1:00 | 100 | 1000 | 1:00 | 53 | 1:00 | 100 | 1000 | 1:00 |
| 45 | 1:00 | 100 | 1000 | 1:00 | 54 | 1:00 | 100 | 1000 | 1:00 |
| 46 | 1:00 | 100 | 1000 | 1:00 | 55 | 1:00 | 100 | 1000 | 1:00 |
| 47 | 1:00 | 100 | 1000 | 1:00 | 56 | 1:00 | 100 | 1000 | 1:00 |
| 48 | 1:00 | 100 | 1000 | 1:00 | 57 | 1:00 | 100 | 1000 | 1:00 |
| 49 | 1:00 | 100 | 1000 | 1:00 | 58 | 1:00 | 100 | 1000 | 1:00 |
| 50 | 1:00 | 100 | 1000 | 1:00 | 59 | 1:00 | 100 | 1000 | 1:00 |
| 51 | 1:00 | 100 | 1000 | 1:00 | 60 | 1:00 | 100 | 1000 | 1:00 |
| 52 | 1:00 | 100 | 1000 | 1:00 | 61 | 1:00 | 100 | 1000 | 1:00 |
| 53 | 1:00 | 100 | 1000 | 1:00 | 62 | 1:00 | 100 | 1000 | 1:00 |
| 54 | 1:00 | 100 | 1000 | 1:00 | 63 | 1:00 | 100 | 1000 | 1:00 |
| 55 | 1:00 | 100 | 1000 | 1:00 | 64 | 1:00 | 100 | 1000 | 1:00 |
| 56 | 1:00 | 100 | 1000 | 1:00 | 65 | 1:00 | 100 | 1000 | 1:00 |
| 57 | 1:00 | 100 | 1000 | 1:00 | 66 | 1:00 | 100 | 1000 | 1:00 |
| 58 | 1:00 | 100 | 1000 | 1:00 | 67 | 1:00 | 100 | 1000 | 1:00 |
| 59 | 1:00 | 100 | 1000 | 1:00 | 68 | 1:00 | 100 | 1000 | 1:00 |
| 60 | 1:00 | 100 | 1000 | 1:00 | 69 | 1:00 | 100 | 1000 | 1:00 |
| 61 | 1:00 | 100 | 1000 | 1:00 | 70 | 1:00 | 100 | 1000 | 1:00 |
| 62 | 1:00 | 100 | 1000 | 1:00 | 71 | 1:00 | 100 | 1000 | 1:00 |
| 63 | 1:00 | 100 | 1000 | 1:00 | 72 | 1:00 | 100 | 1000 | 1:00 |
| 64 | 1:00 | 100 | 1000 | 1:00 | 73 | 1:00 | 100 | 1000 | 1:00 |
| 65 | 1:00 | 100 | 1000 | 1:00 | 74 | 1:00 | 100 | 1000 | 1:00 |
| 66 | 1:00 | 100 | 1000 | 1:00 | 75 | 1:00 | 100 | 1000 | 1:00 |
| 67 | 1:00 | 100 | 1000 | 1:00 | 76 | 1:00 | 100 | 1000 | 1:00 |
| 68 | 1:00 | 100 | 1000 | 1:00 | 77 | 1:00 | 100 | 1000 | 1:00 |
| 69 | 1:00 | 100 | 1000 | 1:00 | 78 | 1:00 | 100 | 1000 | 1:00 |
| 70 | 1:00 | 100 | 1000 | 1:00 | 79 | 1:00 | 100 | 1000 | 1:00 |
| 71 | 1:00 | 100 | 1000 | 1:00 | 80 | 1:00 | 100 | 1000 | 1:00 |
| 72 | 1:00 | 100 | 1000 | 1:00 | 81 | 1:00 | 100 | 1000 | 1:00 |
| 73 | 1:00 | 100 | 1000 | 1:00 | 82 | 1:00 | 100 | 1000 | 1:00 |
| 74 | 1:00 | 100 | 1000 | 1:00 | 83 | 1:00 | 100 | 1000 | 1:00 |
| 75 | 1:00 | 100 | 1000 | 1:00 | 84 | 1:00 | 100 | 1000 | 1:00 |
| 76 | 1:00 | 100 | 1000 | 1:00 | 85 | 1:00 | 100 | 1000 | 1:00 |
| 77 | 1:00 | 100 | 1000 | 1:00 | 86 | 1:00 | 100 | 1000 | 1:00 |
| 78 | 1:00 | 100 | 1000 | 1:00 | 87 | 1:00 | 100 | 1000 | 1:00 |
| 79 | 1:00 | 100 | 1000 | 1:00 | 88 | 1:00 | 100 | 1000 | 1:00 |
| 80 | 1:00 | 100 | 1000 | 1:00 | 89 | 1:00 | 100 | 1000 | 1:00 |
| 81 | 1:00 | 100 | 1000 | 1:00 | 90 | 1:00 | 100 | 1000 | 1:00 |
| 82 | 1:00 | 100 | 1000 | 1:00 | 91 | 1:00 | 100 | 1000 | 1:00 |
| 83 | 1:00 | 100 | 1000 | 1:00 | 92 | 1:00 | 100 | 1000 | 1:00 |
| 84 | 1:00 | 100 | 1000 | 1:00 | 93 | 1:00 | 100 | 1000 | 1:00 |
| 85 | 1:00 | 100 | 1000 | 1:00 | 94 | 1:00 | 100 | 1000 | 1:00 |
| 86 | 1:00 | 100 | 1000 | 1:00 | 95 | 1:00 | 100 | 1000 | 1:00 |
| 87 | 1:00 | 100 | 1000 | 1:00 | 96 | 1:00 | 100 | 1000 | 1:00 |
| 88 | 1:00 | 100 | 1000 | 1:00 | 97 | 1:00 | 100 | 1000 | 1:00 |
| 89 | 1:00 | 100 | 1000 | 1:00 | 98 | 1:00 | 100 | 1000 | 1:00 |
| 90 | 1:00 | 100 | 1000 | 1:00 | 99 | 1:00 | 100 | 1000 | 1:00 |
| 91 | 1:00 | 100 | 1000 | 1:00 | 100 | 1:00 | 100 | 1000 | 1:00 |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 11: The Pride Of Floyd Landis

Realizing a dream not only requires phenomenal personal effort, it requires the acceptance of a phenomenal group effort

By Allen Lim PhD

When I watched Floyd cross the line today, I had to take a moment and step out for some air. So I got on my bike and rode up the mountain away from the team hotel. About a mile down the road I got a call from Robbie Ventura who was at the finish. Robbie could barely talk he was so choked up, which got me all choked up. He told me that when he saw Floyd, the look in his eyes...well, the look was something he had never seen before. The man was so proud.

At dinner tonight, we all lifted our glasses for the man, for Floyd. He was initially coy, perhaps surprised or uncomfortable with all of the attention. But then his eyes smiled brightly and I saw what Robbie was talking about – a lifetime of effort accumulating in a singular moment of self-respect. And as we celebrated I marveled at how present everyone was to this moment. As if time had just stopped.

It struck me in that moment, that realizing our dreams not only requires phenomenal personal effort, it requires the acceptance of a phenomenal group effort. I realized that Floyd's initial shyness at accepting the congratulations of so many was the same hesitation that any of us have when we ask anything of anyone.

We live in a culture where we're taught that asking is a sign of weakness. That being self-reliant, pulling harder on one's bootstraps, and going it alone is the only way to prove our worth. But so many people have come together to help see this moment happen. To see this yellow gown bestowed upon our leader. So many people share in this dream, simply because he asked.

There names are, Bert Grabsch, Robbie Hunter, Nicolas Jalabert, Axel Merckx, Koos Moerenhout, Alex Moos, Victor Hugo Pena, Miguel Angel Perdiguero, Andy Rihs, John Lelangue, Juan Fernandez, Jacques Michaud, Adriano Baffi, Monika Zuercher, Georges Luechinger, Denise Demir, Markus Sugg, Jose Luis Boente, Moises Leboso, Moises Leboso Jr., Juan Pujol, Jose Teixeira, Freddy Viaene, Werner Lenk, Modesto Perez, Cyrille Perrin, Antonio Rodriguez, and Gisbert Kunz. They all raised their glasses tonight. These are the names that make up the team here in France, brought together to support one man, who's here to support this entire family. And right now, we are all so proud.

Below is a summary of today's data. Again, Floyd has achieved new maximum values in almost every metric we measure. Compared to last year, however, Floyd has still not yet hit the same peaks. Of course, he also played a tactically conservative race, following wheels, and waiting for the simple attrition of the climbs to wear people out. There wasn't a single climb that was exceptionally difficult. In fact, the climbing speeds or V.A.M rates (vertical ascent in meters per hour) are significantly lower this year compared to last. But the simple number and duration of climbs took its toll and while everyone will be tired tomorrow, some of the hardest racing is still to come.

| Name: <u>Floyd Landis</u> | | Stage: <u>11. Tour de France (11th de descente) 216.1 km</u> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---------------------------|---|--|------|--------------|-------------------|----------|---------|------------|--------------|--------------|--|--|------------|--|------------|-----|------|-------|-----|-------|-----|------|-----|-----|-----|----------|--|--|--|--|-----------|---------|----------|---------|---------|---------|--------------|------|-----|-----|------|------|-------|-------|-----|-----|-----|----|-------|-----|-----|------|------|-------|-------|-----|-----|-----|----|---------|-----|-----|------|------|-------|-----|-----|-----|-----|----|---------|-----|-----|------|------|-------|-------|------|-----|-----|----|
| Stage Race: <u>5</u> | Time: <u>10h:55</u> | Date: <u>Thursday, July 13th</u> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| UCI Race: <u>1</u> | Time: <u>10:18:57</u> | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | <table><thead><tr><th colspan="4">Average Power (W)</th><th colspan="4">Time (h:m:s)</th><th colspan="2">Climb Data</th><th>4 of Atmos</th></tr><tr><th>Max</th><th>Mean</th><th>Power</th><th>Eff</th><th>Early</th><th>Mid</th><th>Late</th><th>End</th><th>Sum</th><th>Max</th><th>Altitude</th></tr><tr><th colspan="4"></th><th>Early (h)</th><th>Mid (h)</th><th>Late (h)</th><th>End (h)</th><th>Sum (h)</th><th>Max (m)</th><th>Altitude (m)</th></tr></thead><tbody><tr><td>Team</td><td>243</td><td>218</td><td>2478</td><td>2418</td><td>158.8</td><td>156.8</td><td>152</td><td>119</td><td>122</td><td>22</td></tr><tr><td>Floyd</td><td>229</td><td>211</td><td>2277</td><td>2127</td><td>141.1</td><td>138.8</td><td>141</td><td>117</td><td>106</td><td>10</td></tr><tr><td>Stevens</td><td>178</td><td>214</td><td>2411</td><td>2090</td><td>158.8</td><td>159</td><td>222</td><td>249</td><td>252</td><td>22</td></tr><tr><td>McQuinn</td><td>267</td><td>218</td><td>2428</td><td>2412</td><td>121.6</td><td>166.8</td><td>67.9</td><td>116</td><td>177</td><td>45</td></tr></tbody></table> | | | | Average Power (W) | | | | Time (h:m:s) | | | | Climb Data | | 4 of Atmos | Max | Mean | Power | Eff | Early | Mid | Late | End | Sum | Max | Altitude | | | | | Early (h) | Mid (h) | Late (h) | End (h) | Sum (h) | Max (m) | Altitude (m) | Team | 243 | 218 | 2478 | 2418 | 158.8 | 156.8 | 152 | 119 | 122 | 22 | Floyd | 229 | 211 | 2277 | 2127 | 141.1 | 138.8 | 141 | 117 | 106 | 10 | Stevens | 178 | 214 | 2411 | 2090 | 158.8 | 159 | 222 | 249 | 252 | 22 | McQuinn | 267 | 218 | 2428 | 2412 | 121.6 | 166.8 | 67.9 | 116 | 177 | 45 |
| Average Power (W) | | | | Time (h:m:s) | | | | Climb Data | | 4 of Atmos | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Max | Mean | Power | Eff | Early | Mid | Late | End | Sum | Max | Altitude | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | | | Early (h) | Mid (h) | Late (h) | End (h) | Sum (h) | Max (m) | Altitude (m) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Team | 243 | 218 | 2478 | 2418 | 158.8 | 156.8 | 152 | 119 | 122 | 22 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Floyd | 229 | 211 | 2277 | 2127 | 141.1 | 138.8 | 141 | 117 | 106 | 10 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stevens | 178 | 214 | 2411 | 2090 | 158.8 | 159 | 222 | 249 | 252 | 22 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| McQuinn | 267 | 218 | 2428 | 2412 | 121.6 | 166.8 | 67.9 | 116 | 177 | 45 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 12: Tough Day

Despite the lack of climbs, the pace and heat took its toll

By Allen Lim PhD

Despite not having any major climbs longer than 15 minutes today, the speed and the heat definitely made things difficult. At an average power output of 244 watts and just under 4,100 kcals burned, the overall intensity and load today was only slightly less than the first day in the Pyrenees (stage 10). Unlike that first day, however, the variability in power and overall speeds were significantly higher today.

When the power profile is less steady, the strain on the body is much greater. It's similar to the way that stop and go traffic with continuously high revs is hard on your car and bad for gas mileage. But unlike stop and go traffic, the speeds today were extremely fast. So imagine jerking back and forth in a car as the driver continuously accelerates and decelerates all above the going highway speed limit. In a crude way, that's what it probably felt like for Floyd today.

Now imagine doing that in a convertible on an extremely clear, hot, and sunny day. Not that I laughed, but when I saw Floyd come to dinner tonight he had this boiled lobster like glow to him. Perhaps all that yellow he had on today was reflecting more rays.

Another contributing factor to the hard day was some very aggressive riding by other teams that put Phonak on the offensive. Many of the attacks were short and volatile and so it's not so surprising that Floyd hit new maximum values for his best 5-minute and 10-minute power output at 465 and 426 watts, respectively. While they are the highest we've seen in this year's Tour, they're still lower than the best 5-minute of 478 watts and 435 watts we observed last year. So just like yesterday, the stages are getting harder, but the most difficult days are still likely to come.

One last note, in the final 2 kilometers, Floyd threw 7 surges over 10 watts per kilogram of body weight. For Floyd, that type of an effort is a full on sprint. And it's not like Floyd is exactly known for his sprinting abilities. I've actually never seen him put those many top end efforts in the final kilometers of a stage. I think Floyd was equally surprised. The expression on his face when he asked, "did you see that finish man," was the same kind of expression someone has when they asked if you saw the road kill down the road. Still, the fact that Floyd had the motivation and energy to follow the sprinters at the end as they duked it out is a really good sign. Other than that, all is good at the Phonak camp. And that yellow bike. Ya, it's pretty cool.

| | | | | | | | | | | | | |
|---------------------------|-------------------|----------------------|--------------------------|--|--------------------------------|--------------------------|------------------------|----------------|--------|--------------|-----|----|
| Name: <u>Floyd Landis</u> | | | | Stage: <u>12. Euzière to Carcassonne</u> | | | | | | | | |
| Stage Pkts: <u>20</u> | | Time: <u>4:28:53</u> | | Date: <u>Friday, July 14th</u> | | | | | | | | |
| GC Place: <u>1</u> | | Time: <u>5:35:10</u> | | | | | | | | | | |
| Final Results | Average Power (W) | | Work (Kilojoules) / Time | | Time (Min) Distributed Between | | | Best Power (W) | | # of Attacks | | |
| | Moving | Rolling | Power | Feet | Easy to Fairly Hard | Fairly Hard to Very Hard | Greater Than Very Hard | 5 min | 10 min | | | |
| | Today | 214 | 285 | 1890 | 45.4% | 165.2 | 78.3 | 59.6 | 445 | | 331 | 18 |
| | Total Average | 221 | 236 | 1492 | 1399 | 180.2 | 77.7 | 75.5 | 465 | | 331 | 31 |
| | Minimum | 195 | 214 | 2624 | 1682 | 154.4 | 35.9 | 22.2 | 360 | | 252 | 22 |
| Maximum | 267 | 314 | 4520 | 7619 | 220.8 | 166.4 | 17.9 | 465 | 377 | 48 | | |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 13: Hot

The guys were slower, thirstier and more miserable thanks to the warm temps that filled the day

By Allen Lim PhD

In my expert opinion it was hot - really, really, really hot. A Claustrophobic, oppressive, and unkind hot. The kind of hot that makes you want to curl up under whatever patch of shade you can find and suck on a Popsicle. Except you don't have a Popsicle and if you did it would just melt all over you before you could even lick it. And then you'd just be one hot sticky mess. That's how the guys felt and looked when they came across the line today. Of course, my training makes me an expert at recognizing frustrated melted Popsicle heat syndrome.

We could quantify the physiological effect of this type of heat in many ways since heat, like power, is just another form of energy. I was actually planning on transmitting our GPS data to a geographical information system network so we could get real time information about the wind, humidity, temperature, and radiation on the course. My intent was to overlay this data with Floyd's power profile to produce a second to second animated thermograph. Since we know his gross mechanical efficiency, his body surface area, and the heat of vaporization of sweat (0.56 kcals per ml evaporated) it would have been easy to calculate the net thermal heat exchange via conduction, convection, radiation, and evaporation given all the other known variables. But, only after quantifying his total fluid consumption and body mass change over the race. Unfortunately, Floyd didn't want to step on the scale today, I think after someone made a comment about how well the I-Shares logo stood out on his shorts. So I got screwed on that project. But I guess the lesson is, if anyone ever asks you if his or her butt logo makes him or her look fat, in the name of science, just say no!

So to answer the question that's probably on everyone's mind; that question, of course, being, what kind of impact did the heat play on the riders today, I'll just have to assume that the following was due to the heat. First, they were all a lot slower (slowest flat stage so far). Second, they had to drink a ton (at least 20 bottles for Floyd from what he recalls). And third, they were miserable, which is of course relative. After all, the podium girls are hot everyday and when you realize that, you just don't feel as bad.

Actually, today was really unique as the average power was the lowest it's been all Tour at a measly or perhaps toasty 178 watts. Actually, today's average power was only 14 watts higher than the ride into Paris last year. And that day, those guys were drinking champagne, not Gatorade. In any case, the time spent between zero watts to a somewhat hard intensity was the greatest it's been all Tour while the time spent above a very hard intensity was the least it's been for the Tour. Finally, the power profile was also the steadiest it's been all Tour. What all of this tells us is that the guys were crawling today. And with the limited data I have, once again, I can only assume that this was due to the heat, which at one point caused the thermometer in the car to read 42 degrees Celsius or 108 °F.

Tactically, the guys weren't going to do diddly about the breakaway. It's like the movie, "The Sandlot," where its just way too hot to play baseball and the guys go to the pool and in a moment of true genius, Michael "Squints" Palledorous puts the move on Wendy the lifeguard. Except that, the guys couldn't go swimming today. And I'm not exactly sure if anyone kissed Wendy. Instead, they actually eased up near the end to give Oscar P the jersey - a friendly game of hot potato. It's a good move. There's no reason we need to burn anything before the Alps.

Below is a summary of today's data. And since you probably can't read it, you'll just have to trust me. Floyd moved slowly and it was hot.

| | | | | | | | | | | |
|---------------------------|-----------------------|---|--------------------------------|------|----------------|--------------|----------------------|-----|-----|----|
| Name: <u>Floyd Landis</u> | | Stage: <u>13, Highers Mediterranean to Montevideo</u> | | | | | | | | |
| Stage Power: <u>70</u> | Time: <u>4:54:33</u> | Date: <u>Saturday, July 15th</u> | | | | | | | | |
| GC Pace: <u>2</u> | Time: <u>59:57:03</u> | | | | | | | | | |
| | Average Power (W) | | Time (Min) Distributed Between | | Peak Power (W) | # of Attacks | | | | |
| | Minute | Pedaling | Power | Time | 5 | 30 | | | | |
| | | | | | 75% | 75% | Greater Than 70 W/kg | | | |
| Flat | 178 | 217 | 1292 | 1883 | 276.5 | 15.9 | 148 | 288 | 14 | |
| Total Average | 218 | 206 | 2920 | 1828 | 189.9 | 75.0 | 33.5 | 414 | 309 | 29 |
| Minimum | 178 | 217 | 1292 | 1883 | 15.9 | 15.9 | 15.9 | 148 | 288 | 14 |
| Maximum | 267 | 314 | 1830 | 2017 | 276.5 | 306.5 | 47.9 | 365 | 371 | 11 |

Created by ©Allen Lim

Bicycling

Powerfeed Stage 14: The Daily Grind

The daily routine is well...so routine I don't even know where we are anymore.

By Allen Lim PhD

As exciting as it is to be part of the Tour, there's very little glamour about the daily grind. This morning at breakfast, it was obvious that the long days are beginning to wear on both the riders and staff. It was quieter and everyone looked a bit tired. Not the "I can't go on" tired, but more like the, "I wish I could take a nap...and maybe even suck my thumb...with a pillow between my knees." You know, that kind of tired.

Each morning the staff typically wakes up at 6:30 to 7:00 am, for breakfast at 7 or 7:30. Gisbert, our chef is usually up a little earlier getting the race muesli (a mix of organic oats, yogurt, nuts, dried fruit, fresh fruit, preserve, honey, & cream), assorted cereals (ranging from Special K to Sugar Smacks), pasta, rice, sauce, cheese, omelets, yogurt, coffee, tea, milk, soy milk, juice, chocolate croissants, apple strudels, fresh fruit, and assorted vitamins and supplements. Of course, most of this food is for the riders. For the staff the norm is some coffee, a croissant, and maybe a little muesli.

In any case, breakfast normally lasts about 30-minutes and then the mechanics and soigneurs are out getting everything ready for the day. Tires are pumped and bikes are inspected. Cars are washed, vacuumed, and racked with spare bikes (Floyd has two on each of the two follow cars). The van is racked with race bikes (Floyd's race bike is identified with a red saddle). The bus is disinfected (how this is done I have no idea). Vehicles are fueled. Two hundred and fifty to three hundred bottles with a carbohydrate and electrolyte solution are filled. Coolers are packed with these bottles as well as water, Coke, Red Bull, and Orangina. Bottles of frozen water are hammered apart for ice. Sandwiches for staff (lunch) and riders (post-race) are prepared. Race food ranging from fresh fruit, to little sandwiches, pastries, energy bars, and candy bars are wrapped in a special paper foil that makes it easier for the riders to open. Thirty musette bags filled with two bottles, two gels, two bars, two candy bars, two pastries, and some fruit are made. Luggage is brought down and packed in the truck. Clean laundry is sorted and distributed. Rain bags (individual bags filled with each rider's rain cape, spare shoes, change of race clothes, gloves, and extra helmet) are inspected and loaded. Food is loaded in the camper. Finally, race coordinates and deviations are programmed in each vehicle's GPS.

Once all of this is done, the mechanics truck, camper with food, and one soigneur van head to next hotel at about 10 am to unload bags, prepare kitchen, and organize rooms. Two caravan cars, one media car, the bus, and a soigneur van sit waiting to take the riders to the race at around 10:30 am.

The riders are typically scheduled to wake up an hour later and usually trickle in at about 8 or 8:30 for breakfast. The exception is Floyd who every morning of the Tour has been up with the staff and is normally at breakfast with them. The funny thing is that we have separate tables set up for staff and riders since our staff is so large and because the rider's table has so much more food on it. It's kind of like the adult table and the kid's table at Thanksgiving. So Floyd always ends up eating breakfast alone at this smaller table filled with way too much food, while 20 staff members all sit at a separate even larger table staring at him. I always give him a nod and he just looks around and shrugs his shoulders. After Floyd eats, he goes back to sleep and usually waits until the last minute to get on the bus in normal street clothes while the other riders all suit up before. Floyd's not big on chamois time.

The riders usually get to the race an hour before the start and have a meeting with the team's directors in the bus about the day's tactics. Then they hang out in the bus and smack talk behind the very darkly tinted windows. Sometimes, I leave them things to ponder, like web print outs of articles from "The Onion," Jack Handy quotes, and pictures of various types of American and European mullets. I'm sure this kills lots of time. Or they just get a coffee in the Tour Village. You can also get a hair cut and samples of local cuisine in the Village. I plan to get my head completely shaved there while eating a crepe before the final time trial. Somewhere in this time the riders also sign in and about 10 minutes before the start roll to the line. The race starts and grown men suffer for the next 4 to 6 hours.

Back at the next team hotel, we're hauling staff and rider bags into each of their respective rooms after just finishing a 2 to 3 hour drive in gridlock. The unloading and distribution of bags is equally time consuming and involves a lot of sweating and grunting. Except nobody's naked. It really sucks. While the bags are getting done, Gisbert or "Chef Man" as I call him (he calls me "Computer Man"), goes shopping for fresh organic food and prepares the kitchen. He tells me that the most important part of his job is making sure the kitchens are sanitary as food poisoning at the Tour isn't just something Floyd makes fun of, it's actually a very real possibility. At the same time, the mechanics are busy getting their work area together and catching up on anything mechanical they have to do. After the bags are done, I start number crunching and preparing my daily stories while watching the Tour on T.V. If the soigneurs and mechanics are done with their work, we usually all gather in the lobby or one room to watch the race. Almost always, everyone falls asleep while watching.

When the riders get done with the race, they either ride back to the hotel (as happened today) or they shower in the bus, refuel, and settle in for a 30 minute to 2 hour transfer to the hotel depending on where we are staying relative to the finish. The guys head directly to the "Food Room" where more muesli, fresh fruit, sandwiches, cereal, fluid, and all sorts of Scooby snacks are waiting. They then get checked on by the team docs, get a massage, then get any adjustments or rehab work done by our osteopath.

By 8:30 pm the riders are having dinner that starts with pasta, rice, potatoes, or all of the above with eggs, sauce, and fresh Parmesan cheese. Then they have a big salad. Once their done with this, they actually have a normal dinner -- the same dinner that the staff will eventually have about an hour later. Desert is always a possibility as is wine and lots of cheese. We are, after all, in France.

I normally skip dinner to crunch the data from the day and to write these stories. Eventually, everyone goes to bed and we repeat. Nothing changes. I don't even know where we are anymore.

With that in mind, today was another hard day. Go figure. Actually, it was really unique because the stats were very comparable to stage 11 -- the hardest stage so far. Floyd held an average of 265 watts today, only 3 watts less than stage 11. The difference was that today's stage was shorter and over flatter terrain. So the riding was less steady and extremely volatile, especially in the first hour. In fact, today Floyd did more surges above 8 and 10 watts per kg of body weight than any day of the Tour. Those accelerations aren't his forte, so today was a tough one. The nice thing is that Floyd likes it hard, because if it's hard for him, it means everyone is hurting. That all said, tomorrows rest day is really welcome - for all of us.

Name: Flord Lardis Stage: 14. Mindless Grind
 Stage Pace: 53 Time: 4:15:30 Date: Sunday, July 15th
 GC Pace: 2 Time: 58:06:33

| | | Average Power (W) | Wheel (Kilowatt) Power | Time (Min) Distributed Between | | | | Best Power (W) | | # of Attacks |
|------------|--------------|-------------------|------------------------|--------------------------------|------------|--------------|-----------|----------------|-----|--------------|
| | | | | Early Hard | Early Hard | Greater Than | Very Hard | 5 | 10 | |
| Flych Load | Fields | 265 | 318 | 4247 | 4636 | 128.5 | 79.8 | 18.0 | 445 | 15.8 |
| | Turn Average | 228 | 260 | 3034 | 3539 | 105.8 | 76.3 | 14.2 | 317 | 207 |
| | Average | 178 | 217 | 2051 | 2092 | 128.5 | 15.0 | 15.0 | 180 | 252 |
| | Maximum | 20.7 | 11.8 | 1870 | 7013 | 726.4 | 366.9 | 37.0 | 365 | 379 |
| | | | | | | | | | | |

Photographed by Jason Tanaka Blaney at the Lakeside Lounge, NYC

Bicycling

Powerfeed Stage 15: All That Matters is Yellow

Confidence and happiness abound in the Phonak camp

By Allen Lim PhD

Today was a beautiful day. It's hard to describe the emotion here at the Phonak camp so I won't even try. I'll just say that Floyd is happy and confident. We all are.

On the course today he was strong, in control, and had a tactically and physically perfect race. More importantly, the team showed its strength today and proved that they can be there for Floyd when it counts the most. Perdiguer's lead out at the base of L'Alpe d'Huez was perfect. Merckx, making the early break and then his strong work in the final, could not have worked out any better for Floyd. In the end, it was all up to Kloden to do the work as he was down on GC from Floyd and had to fight to try to separate himself from Floyd and the others. But Floyd was simply too strong to be dropped and though he could have gone on the attack, right now, yellow is the only thing that matters.

With that in mind, we had some unfortunate luck with the PowerTap. Floyd got a flat 100km into the race and because of the wheel change we lost data from that point on. So we missed some of the most critical data of the Tour, it's a real bummer. We dropped the keys in molten lava...again. But, yellow is the only thing that matters.

In an attempt to salvage or resurrect some data, I went and talked to Michael Georges of Matsport, the man responsible for timing this entire race. He took me on a tour earlier today and showed me how all the timing systems work- the chips on the bicycles, the timing sensors on the road, and the GPS network that gives them real time info on time gaps. It was computer geek nirvana, not that I was impressed or anything. Anyway, he told me that he had set up some special sensors at the base of L'Alpe d'Huez today so that he could get everyone's time up the mountain. It was just something he was interested in. So I tracked him down and he has offered to give me all the splits. The terrain profile on L'Alpe d'Huez is so detailed that it will be possible to get a fairly accurate estimate of Floyd's power on the climb. It will take me some extra time tonight to crunch those numbers, but hopefully I'll be able to report a bit on that tomorrow.

As for tomorrow, my thought is that it will be one of the most important days of the Tour. Yes, today was hard, but we know from the Dauphine earlier this year that the 18.4 km climb up to Toussuire, though not the steepest, will take it's toll, especially after the Galibier (42.8 km, 4.5%), Col de-La-Croix de Fer (22.7km, 6.9%), and Col du Mollard (5.8km, 6.8%). So it's still one day at a time. It's just how dreams work. One day at a time. And right now the dream is yellow.

Bicycling

Powerfeed Stage 16: Perspective

Despite cracking on today's stage, the fire is still there and Floyd isn't going home without a proper fight

By Allen Lim PhD

What do you say after a day like today? Do you say that it's all right when it's actually not? Do you sit around and micro-analyze every detail to find an answer when you'll never really know? Or do you just say it is what it was - a really bad day.

After the chaos of media and spectators died down tonight a few of us stepped out with Floyd for a beer. Rather than being in a fowl mood, Floyd was just happy to be acting like a normal person again. As we sat there, he got a text message from Jonathan Vaughters that got him laughing as he announced, "this is the best thing I've heard all day." Floyd read the message to us. A message that basically said, "I know that everyone is telling you that it's all okay and to keep it in perspective, that it's not the end of the world. But this sucks ass!" Finally, a little bit of truth...today sucked ass.

Everybody is doing the best they can otherwise they'd be doing better. That was one of my lines for the evening. Floyd's response was something on the order of, "What kind of crack are you on?" I replied, "Well at least we'll have something to do for next year." His answer was short and sweet, "I'm going to kick your ass." So I'm happy to report that despite cracking on today's stage, the fire is still there and this boy isn't going home without a proper fight.

Long story short, he didn't feel good from the get go. Yesterday was a big effort and no matter what you do, sometimes a big effort means the next day isn't going to be so pretty. There's no amount of eating, sleep, magic potion mixing, or aromatherapy that is going to fix that in under 18 hours, especially after 2 weeks of racing. Floyd didn't bonk, he wasn't dehydrated, and his hip didn't feel bad. It just wasn't there for him. He felt awful and spent most of the day in damage control.

For every rider at the Tour, bad days just happen. In the end, which day your number gets called is almost luck of the draw. If today's terrain had been even a notch lighter, he would've been all right. Why did it have to be today? I don't know. But not everything is due to random chance or lives in the realm of mystery. We're better than that and hopefully in time smarter than that. All we know now, is the Tour is unrelenting. It's like no other test of human strength and determination and at times it's just simply unreasonable, not just for Floyd but also for everyone who suffered up to Toussuire today. We'll figure it out though. He'll figure it out.

In time, he'll understand why an otherwise reasonable average power output of 259 watts felt like he was being beat by a wrecking ball. The previous and current days nutritional, energy, and fluid intake will be re-assessed. Files will be compared and analyzed. The latest scientific journals will be dissected. Ideas will come and plans will be made, tested, and re-tested. And sometime next week Floyd will be back at home drinking a beer in his yard with a big old smile on his face. So rather than analyze his power profile and GPS data from today, I'm just going to enjoy thinking about next week and the possibilities that still remain between. Cause this race isn't over yet. He's just angrier now and the dream remains - raw, unsympathetic, and once again provoked.

Yes, today sucked. And yes, maybe it doesn't help to try and keep it in perspective. But, I am beaming with pride for Floyd tonight. Because, without even knowing it, he's got it in perspective - he's made it all okay. He's wiped off the dirt and is getting on with it. I mean we all know that the world loves a winner, but at the end of the day, hopefully your friends, your family, well, hopefully, they only love you. That's Power.

Bicycling

Powerfeed Stage 17: Redemption

Today Floyd proved that he has it. Today he gave everyone a reason to believe.

By Allen Lim PhD

I'm so overwhelmed right now, I can barely even write. This morning he was so angry...so mad at himself. He had the music cranked to max as he paced around his tiny hotel room like a wild animal, foraging for his belongings so he could pack his suitcase for the transfer. His appetite for redemption was so raw and you could see his thirst for blood as he proclaimed, "I'm the strongest guy in this race! And yesterday was crap! I may lose this Tour, but it's going to cost them!"

I stood there with a scale and a piece of paper in my hand. "I need to weigh you," I said. His attention suddenly shifted and with one quick shove he slammed the bed up against the wall to open up some floor space. "Here, we've got some room now and you can sit on the bed," he said. I put the scale on the floor and he threw me a small book. It was a book of Jack Handy quotes that I asked Scott Thompson from Quality Bicycle Products to send. "Scott sent these," he said proudly. "I know," I said. And then we just sat there and read each other Jack Handy quotes for the next 15-minutes. He laughed louder than I think I've heard him laugh all Tour.

I showed him the piece of paper. On one side was every reason I could think of for his bad day. I asked him what he thought and his answer was simple. "I just sucked yesterday but it's not going to happen again." On the other side was a list of power values showcasing his best moments at this year's Tour. I showed him the numbers and he once again proclaimed, "I'm the best guy here. Today, I'm going ape shit. Today, I'm going to win."

We took his weight. I told him good luck then headed off for the finish. But he's never really needed luck or any sentimental "Chicken Soup" for his soul to get over a bad day. Just a bit of humor to lighten the mood, loud music, a wide-open road with nothing to lose, and the unyielding belief that he's the best in the world. Well, whatever it takes, today he proved that he has it. Today he gave everyone a reason to believe.

- 5 hours 23 minutes and 36 seconds.
- Covering 200.5 kilometers (130 km alone in the wind).
- At a speed of 37.175 km/hr.
- Averaging 281 watts when moving for the whole ride and 318 watts over the last two hours.
- Averaging 324 watts while pedaling for the whole ride and 364 watts over the last 2 hours.
- At an average cadence of 89 rpm.
- Transferring 5,456 Kjoules of energy to his Cycleops PowerTap.
- Taking, no joke, a total of 70 water bottles (480 ml each) from the car to keep himself cool and hydrated.
- Attacking about a quarter of the way up the Col des Saisies for 30 seconds at 544 watts, which settled into a 5-minute peak of 451 watts, which continued for 10 minutes at an average of power of 431 watts, and left everyone in his dust after 30 minutes at an average power of 401 watts.
- Spending 13.2% of his time or 43 minutes coasting like a rocket on the descents and another 60% between 4 to 7 watts per kilogram of body weight (aka, the pain cave).
- Holding onto 373 watts over the Col de Joux-Plane.
- Hitting a max speed of 83.7 km/hr (51.9 mph) and flying like a Phoenix on his way to the most incredible moment in sports I have ever witnessed.

Bicycling

Powerfeed Stage 18: The Race Isn't Over Yet

The last 24 hours have literally been the busiest of the Tour as Floyd prepares for the final time trial

By Allen Lim PhD

It was another hot day today and Floyd didn't do anything but hang out in the peleton, eat, and drink. Call it 200 km of fueling up for the final throw down of the Tour. So there's really not that much to report. The power file looked like a stroll in the park compared to yesterdays data and though Floyd was feeling tired this morning, today's ride wasn't bad for recovery. If only the bad day was today. After yesterday's amazing ride, it's almost better that things worked out the way they did. Well, maybe not. But, sometimes you never really know what you're made of until you've hit bottom.

Which, of course, brings me back to yesterday. I can't stop thinking about it. This morning I talked to Floyd about the day and he was still beaming from it. As we reviewed the data, both of us were impressed at the level of effort the day required. At the same time, however, we both also realized that the ride was well within his capacity. That he had done rides this big in training. Yes, it was a truly amazing effort, but both of us already knew how strong he is. The day was special because he finally got to show the world that strength. He found himself again and he never stopped believing. That's how he won that race. It was because he believed and no one else did.

With that in mind, I'd thought it'd be interesting to share a few more stats from yesterday -- specifically, the power outputs on the climbs.

Col des Saisies (6.4%): 395 watts in 36:55.

Col des Aravis (7.1%): 371 watts in 16:49.

Col de la Colombiere (5.8%): 392 watts in 27:45.

Cote de Chatillon-sur-Cluses (4.9%): 374 watts in 11:07.

Col de Joux-Plane (8.5%): 372 watts in 37:34. As a point of reference, these power outputs were very similar to the average power outputs over the 5 climbs on Stage 11 where Floyd first took yellow. More interesting, is the fact that over the course of the Tour, Floyd's best power output for 10-minutes didn't actually happen in the race. It actually happened on Monday's rest day when he decided to open it up on a hill near the hotel and cracked 460 watts. He was doing similar efforts in training before Georgia and before the Tour. So long story short, the conservative first two weeks was, in hindsight, not really true to his talent or style and I'm certain that this will be the last time we see Floyd do what everyone thinks he's suppose to do.

Now everything hinges on the time trial. While we are all confident that he will rage tomorrow, we're not taking anything to chance. This evening we had what looked like every engineer from BMC working on Floyd's time trial bike. It is truly impressive the quick and superb work these guys have done to re-spec Floyd's bike since the UCI rule change that forced him to lower his bars and the accident in the first long time trial. In addition to the bike, the directors drove the course tonight and it looks like the first 10-15 km is fairly technical, the middle 20-25 km is where most of the time will be made, and the final 10-15 km is fairly flat to downhill which will tend to keep the time differentials low. Floyd will be pre-riding the course in the morning to take a look for himself. In the meantime, we've got different pacing models built up for each 5 km section and have some pretty good idea about what he'll be able to hold and need to hold for Yellow. This thing isn't even close to over yet and the last 24 hours have literally been the busiest of the Tour. This staff has really rallied behind Floyd's incredible ride and we'll be doing everything we can to ensure he has the best ride of his life. What we all know though is that tomorrow he'll be carrying us. Like I said at the beginning of this Tour, "this guy is going to win the Tour."

Bicycling

Powerfeed Stage 19: Thanks Floyd

What you'll suffer through to make the dream happen

By Allen Lim PhD

As much as we all believed that Floyd would win, when he crossed the line today I couldn't believe it was happening. The moment was so unreal, so unlike anything I was prepared for, and at the same time so normal and expected. It left me in a daze. It's really difficult to try and express how everyone feels, how I feel. Of course, we're all happy, relieved, and excited. But I'm also feeling exhausted and pensive. It has been such a long and hard road to get here. Many, many bad days - more than just the one Floyd had last Wednesday. Now all balanced by this Yellow prize. Last year our joke was that the Tour was 99 One, which stood for 99% bullshit, 1% pure magic. We've been living and working for the magic ever since and it really does feel great to turn all that crap into gold. A lot better than losing, I'll tell you that much. But I can't help ignoring the other 99 right now. Because it was all that misery to get here that makes it worth it.

We were up at 6 this morning. No change in the routine. Gisbert squeezed fresh orange juice, just like he had every morning of the Tour and brought out a bucket of muesli. The pasta was made, eggs scrambled, and chocolate croissants spread out. Floyd was again, the first one at breakfast. The room felt really quiet, the way it does when the world isn't quite up yet. We talked more about the power he did on Stage 17 and Floyd got a kick out of it. I told him that I thought he'd do a 1:08:40. He thought it was too fast. By 7:45 am, we were out the door to check out the time trial course.

Keeping with tradition, Robbie Ventura and I joined Floyd to pre-ride the course. Floyd was in a great mood, just happy to be on his bike. It rained, but it didn't change how much fun we were having. We were like kids riding our bikes -- very far from the seriousness of the day ahead. Along the course the fans were already out in force and it wasn't even 8:30 am. As we rode along, everyone on the side of the road lit up with "Allez Floyd," or "C'est Landis." It gave me goose bumps. He's become an overnight legend here in France.

After the ride, Floyd headed back to the hotel for food and a nap. At 3 pm, he was back at the start. The warm-up wasn't hard. He kept it light so that he wouldn't get overheated as the day was already getting close to Popsicle sucking hot. He used an ice vest, drank water and an electrolyte-glucose drink, had two gels, and listened to "Fist of Rage" by Kid Rock many times.

For his Tour winning performance, I estimate his average power output at 394 Watts, about 3.5% better than he did in the final time trial last year, but not as strong as the 410 Watts that he unleashed on the first time trial. It was plenty.

When we got back to the hotel, the champagne, beer, and I don't know what started flowing like a damn broke. The mechanics broke out in song while putting yellow handlebar tape on all the bikes and everyone got busy congratulating each other.

I didn't want to miss dinner (as I normally have been to do these stories), so I found an empty room to get this written as quickly as possible. In the middle of putting this piece together, I was surprised to see Floyd side step the celebration outside and join me and my brother who flew in yesterday to help me keep my shit together (it's been a brutal Tour). Floyd was feeling the same way I was. Dazed and perhaps even sad that it's all over. I asked him what he was thinking and how it felt to put on that Yellow jersey. His reply was that he thought that maybe the process was more fun than the victory. It was the most sentimental thing I think I've heard him say all month. I asked, "You think the 99 is better than the One?" He replied, "It's never the same as you imagine."

So maybe it's not all the misery that makes getting here worth it. Maybe we just love the misery - the process of believing so deeply in your dreams that you'll suffer through anything to make them come true. After all, "passion" is to suffer. We've arrived and it really is magical. These memories are searing in my mind like a hot iron. And Floyd is truly the happiest and the proudest I've ever seen him. But there's no way we're putting it in park now. We can't. Cause time just keeps moving forward and though we have no idea what lies ahead, we have no choice but to follow. A new era has begun and a new road lies ahead, once again expectant and impatient. So before we start moving again, before the trucks get packed up and start their way towards Paris and the inevitable future, I just have to say thanks. Thanks Floyd. You've made my dreams...you've made all of our dreams come true.

Bicycling

Powerfeed Stage 20: Just Believe

Floyd is officially the Tour champ, let the party and the thanks begin

By Allen Lim PhD

It's a mob scene here. I can't get anywhere near Floyd right now, let alone his bicycle, so I'm going to file this last report without checking out his victory day stats. What I can tell you is that last year, he averaged 164 watts. I'm willing to bet that today's power profile was exactly the same as last year's for this stage. The only difference is that this year, he's the winner...I told you so.

I'm about to head off to the team party right now so I need to keep this short. In time, I think I'll be able to find the words to express all the emotion that this win has created for myself, Floyd, and the huge team of riders, staff, family, friends, and fans that made this all possible. Right now, I'm just too exhausted to put it into words. So for now I just want to say thanks to anyone and everyone who ever lifted a finger for us. We are indebted to so many people. At the hotel today, seeing all the family and friends who have come into Paris to greet all the riders made that point obvious and clear.

Although the list of people to thank is way too extensive, and I will probably screw this up, I have to thank David Cathcart and CycleOps Power as well as Robbie Ventura for connecting me with Floyd, and starting this crazy ride more than a year and a half ago. Of course, to my friends and family who were always unconditional in their support even if they never really understood what it was I did or whether or not it was a real career. Not to scare you, but I still haven't figured that one out yet. Thanks, to everyone on the TIAA-CREF Cycling Team and all the athletes/students/friends who have taught me more than I could ever teach. In particular, to Michael and Dede Barry, Christian VandeVelde, Alex Candelario, Jesse Bartholomew, and Dr. Shannon Smith - you guys kept me in this game at times when I was ready to pack up and fly home. And finally, to Dr. William Byrnes, my mentor at the University of Colorado at Boulder - not a day went by at this year's Tour that I didn't hear your voice in my head, forcing me to rethink, re-analyze, and suffer over every statistic, idea, or bit of advice I gave Floyd. Thermoregulation...how cool is that.

All of you had one thing common. You all believed. Floyd believed and he made me believe and we all believed together. For a science guy, I have to say, that was the best leap of faith I've ever made. Just believe...anything is possible.

Dr Brendan M Buckley FRCPI FFPATH FFSEM

**The Cork Clinic
Western Road
Cork**

Dr Una May,
Anti-Doping Unit,
The Irish Sports Council,
Block A, *Top Floor*,
West End Office Park,
Blanchardstown,
Dublin 15.

09 June 2006

Dear Dr May

Re:

The analytical findings on urine of an athlete sampled out of competition on September 30th 2005 and analysed in the Drug Control Centre of King's College London and therein coded A382195.

Dear Dr May:

The Authorised Expert Group, convened by the Irish Sports Council to consider the analytical findings detailed above, met on 5th May 2006 and subsequently further considered the data provided to us by the ISC. These data include the results of analysis of three prior and two subsequent urine samples from the same athlete.

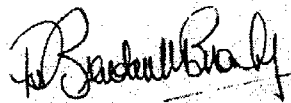
The Authorised Expert Group, having regard to the abnormal results of the tests on sample coded A382195 as well as their relationship to the normal results of prior and subsequent samples, considers that

1. The results deviate significantly from the range of values normally found in humans.
2. Are unlikely to be consistent with normal physiological endogenous production.

3. Are unlikely to be consistent with endogenous production due to a pathological state such as a congenital abnormality of androgenic steroid production, or an androgen-secreting tumour, or any other known pathological condition.

We therefore advise that we concur with the conclusions stated by Professor Cowan in his report of 28th April 2006 to you that the urine sample obtained by you on September 30th 2005 and analysed in the Drug Control Centre of King's College London and therein coded A382195 contained an abnormal concentration of testosterone, consistent with the administration of testosterone or of a substance that raises the concentration of testosterone.

Yours Faithfully



Dr Brendan M Buckley

Consultant Endocrinologist, Bon Secours Hospital, Cork.

Unanimously, on behalf of:

Professor T. Joseph McKenna

Consultant Endocrinologist, St Vincent's University Hospital, Dublin

Dr James Gibney,

Consultant Endocrinologist, The Adelaide and Meath Hospital, Dublin, Incorporating the National Children's Hospital

IRMS Data provided by the WADA accredited Laboratory in Turin, Italy.

GDC00866

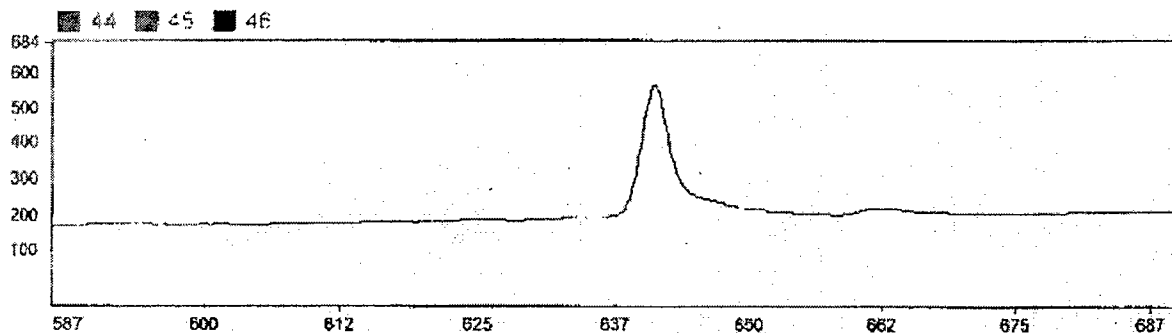
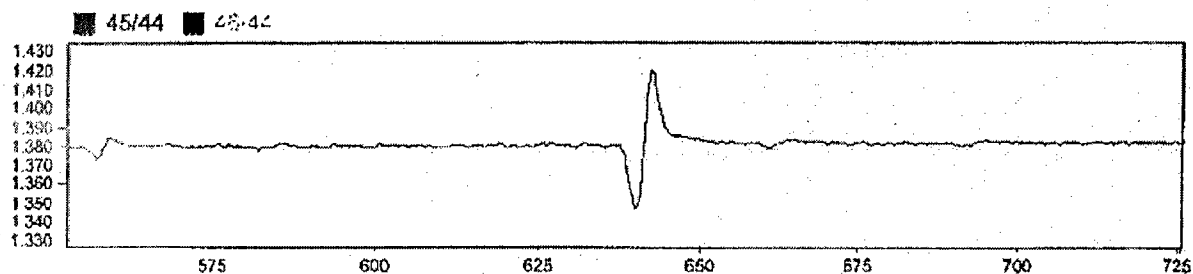
LABORATORIO ANTIDOPING
FMSI
Stazione temporanea T1
Orbassano (TO)

SP5

Sunday, March 19, 2006 21:17:18

C:\DATAT1-RI001\060319\0nd 6_.dxf

| Row | Peak Center | GC Method | AS Sample | AS Method | Identifier 1 | Method |
|-----|-------------|------------------|-----------|-----------|--------------|------------------|
| 5 | 1 | Rt_splitless.gcm | 5 | RI.asm | 0nd 6 | RI_splitless.met |



| Peak Nr. | Component | Rt [s] | Ampl. 44 [mV] | BGD 44 [mV] | d 13C/12C [per mil] vs. VPDB |
|----------|-----------|--------|---------------|-------------|------------------------------|
| 1 | REF. 1 | 87.2 | 4357 | 1.3 | -11.221 |
| 2 | REF. 2 | 125.8 | 4360 | 2.6 | -11.394 |
| 3 | REF. 3 | 166.8 | 4355 | 2.9 | -11.458 |
| 4 | REF. 4 | 206.5 | 4351 | 3.0 | -11.488 |
| 5 | REF. 5 | 445.4 | 4363 | 118.4 | -11.625 |
| 6 | REF. 6 | 495.1 | 4374 | 133.6 | -11.700 |
| 7 | REF. 7 | 641.2 | 374 | 192.0 | -22.255 |
| 8 | REF. 8 | 1052.7 | 4378 | 393.6 | -11.602 |
| 9 | REF. 9 | 1102.5 | 4376 | 385.0 | -11.854 |
| 10 | REF. 10 | 1152.4 | 4357 | 2.7 | -11.542 |
| 11 | REF. 10 | 1202.0 | 4357 | 2.5 | -11.551 |

GDC00867

LABORATORIO ANTIDOPING

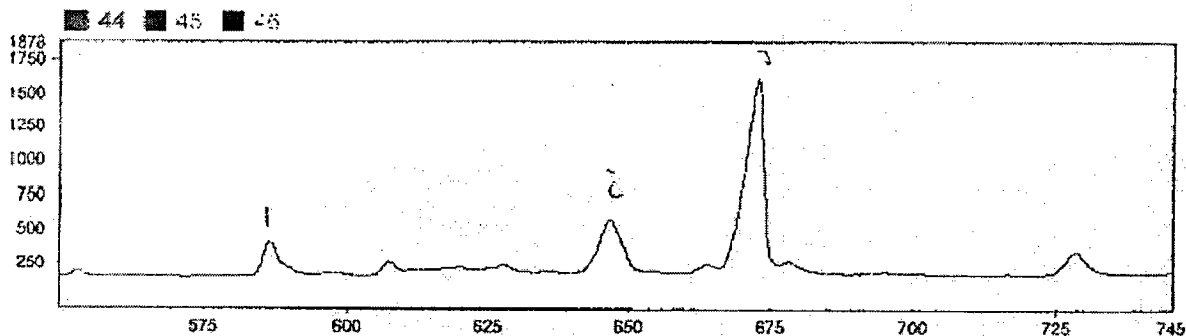
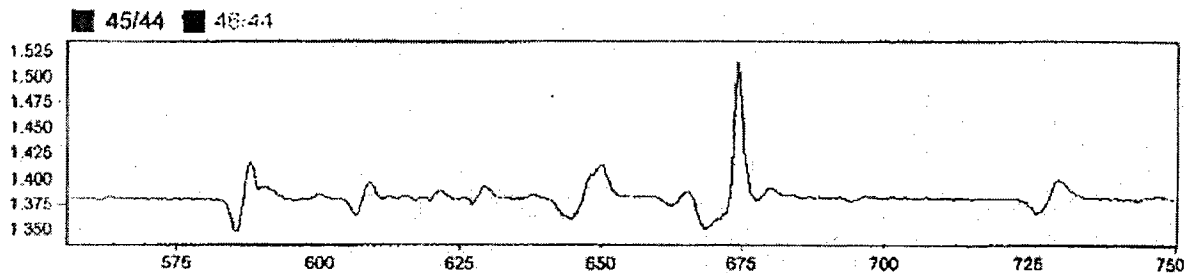
FMSI
Stazione temporanea T1
Orbassano (TO)

Sunday, March 19, 2006 19:35:00

H25

C:\DATAT1-RI001\060319\Lond 1_.dxf

| Row | Peak Center | GC Method | AS Sample | AS Method | Identifier 1 | Method |
|-----|-------------|------------------|-----------|-----------|--------------|------------------|
| 1 | 1 | RI_splitless.gcm | 1 | RI asm | Lond 1 | RI_splitless.mat |



| Peak Nr. | Component | Rt [s] | Ampl. 44 [mV] | BGD 44 [mV] | d 13C/12C [per mil] vs. VPDB |
|----------|---------------|--------|---------------|-------------|------------------------------|
| 1 | REF. 1 | 86.9 | 4300 | 0.9 | -10.905 |
| 2 | REF. 2 | 126.9 | 4315 | 2.1 | -11.175 |
| 3 | REF. 3 | 166.8 | 4311 | 2.5 | -11.302 |
| 4 | REF. 4 | 206.5 | 4311 | 2.8 | -11.365 |
| 9 | REF. 5 | 445.4 | 4326 | 92.6 | -11.520 |
| 10 | REF. 6 | 495.1 | 4333 | 107.5 | -11.700 |
| 11 | 1 - 11 K/L 30 | 586.7 | 274 | 149.2 | -21.453 |
| 12 | 2 - 11 O/L 30 | 646.9 | 399 | 177.6 | -21.556 |
| 13 | 3 - 11 S/L 30 | 673.0 | 1444 | 184.6 | -21.226 |
| 15 | REF. 7 | 1052.7 | 4334 | 207.7 | -11.749 |
| 16 | REF. 8 | 1102.5 | 4339 | 203.3 | -11.832 |
| 17 | REF. 9 | 1152.2 | 4322 | 2.4 | -11.581 |
| 18 | REF. 10 | 1202.0 | 4328 | 2.2 | -11.547 |

GDC00868

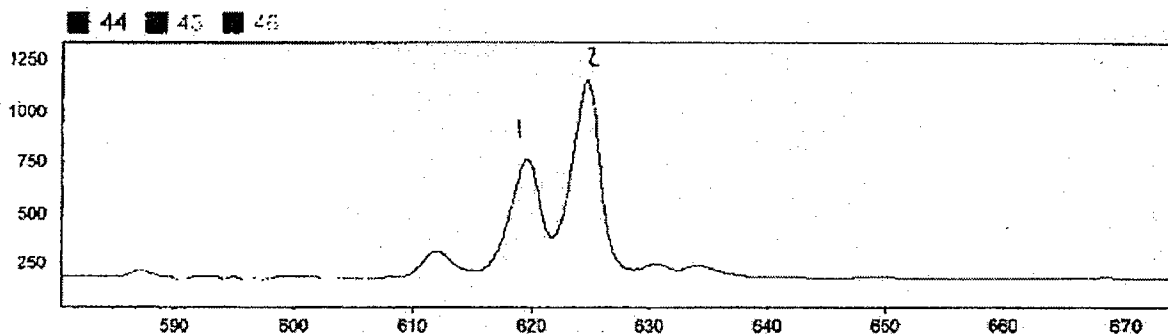
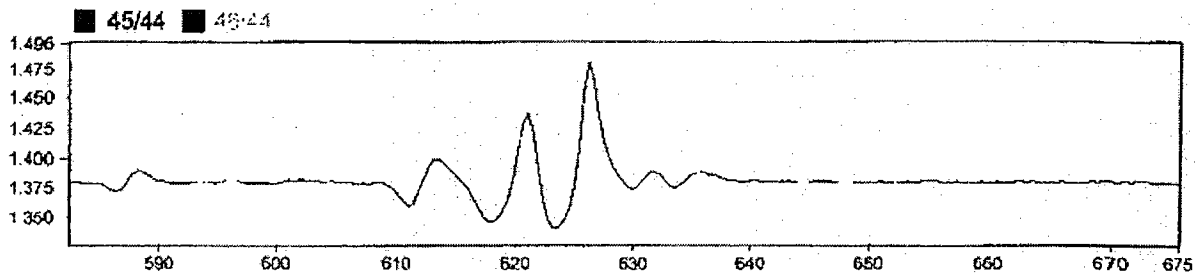
LABORATORIO ANTIDOPING
FMSI
Stazione temporanea T1
Orbassano (TO)

HP5

Sunday, March 19, 2006 20:00:35

C:\DATAT1-RI0011060319\Lond 2_.dxf

| Row | Peak Center | GC Method | AS Sample | AS Method | Identifier 1 | Method |
|-----|-------------|------------------|-----------|-----------|--------------|------------------|
| 2 | 1 | RI_splitless.gcm | 2 | RI.asm | Lond 2 | RI_splitless.met |



| Peak Nr. | Component | RT [s] | Ampl. 44 [mV] | BSD 44 [mV] | d 13C/12C [per mil] vs. VPDB |
|----------|------------|--------|---------------|-------------|------------------------------|
| 1 | REF. 1 | 86.9 | 4329 | 1.1 | -10.888 |
| 2 | REF. 2 | 126.4 | 4327 | 2.4 | -11.129 |
| 3 | REF. 3 | 166.8 | 4326 | 2.7 | -11.188 |
| 4 | REF. 4 | 206.5 | 4326 | 2.8 | -11.225 |
| 10 | REF. 5 | 427.8 | 4382 | 130.7 | -11.460 |
| 11* | REF. 6 | 489.1 | 4415 | 131.0 | -11.700 |
| 16 | 1 - EPI | 619.7 | 556 | 209.4 | -21.375 |
| 17 | 2 - 25.575 | 624.7 | 945 | 209.4 | -22.136 |
| 18 | REF. 7 | 1052.7 | 4348 | 247.2 | -11.569 |
| 19 | REF. 8 | 1102.5 | 4348 | 240.4 | -11.613 |
| 20 | REF. 9 | 1152.4 | 4333 | 2.5 | -11.332 |
| 21 | REF. 10 | 1202.2 | 4344 | 2.3 | -11.326 |

GDC00869

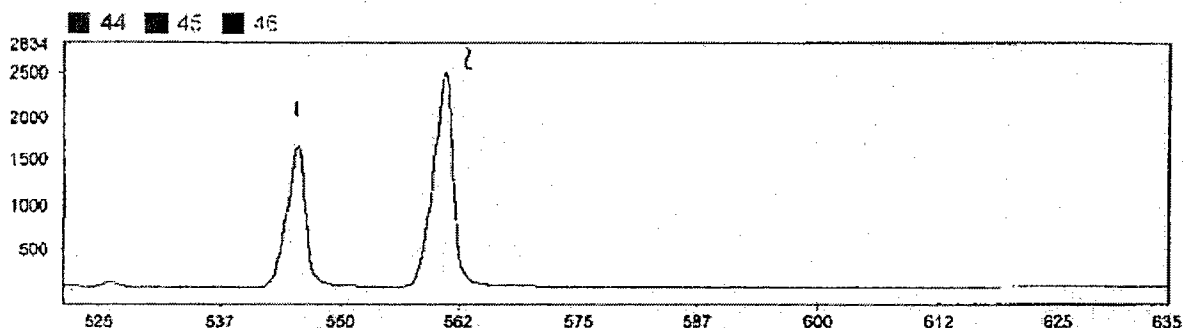
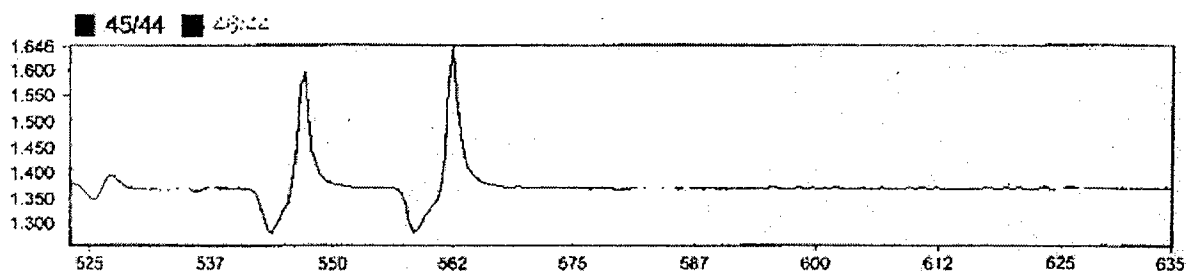
LABORATORIO ANTIDOPING
FMSI
Stazione temporanea T1
Orbassano (TO)

HP 5

Saturday, March 18, 2006 16:16:37

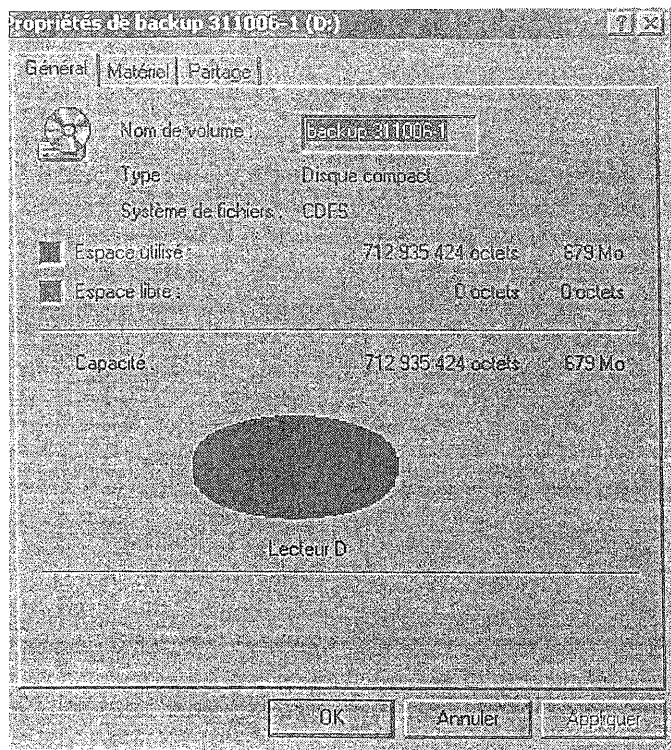
C:\DATAT1-RI001\060318\Iond-4_.dxf

| Row | Peak Center | GC Method | AS Sample | AS Method | Identifier 1 | Method |
|-----|-------------|------------------|-----------|-----------|--------------|------------------|
| 1 | 1 | Rt_splitless.gcm | 3 | RI.asm | Iond-4 | RI_splitless.met |



| Peak Nr. | Component | Rt [s] | Ampl. 44 [mV] | BGD 44 [mV] | d 13C/12C [per mil] vs. VPDB |
|----------|-----------|--------|---------------|-------------|------------------------------|
| 1 | REF. 1 | 86.9 | 4351 | 1.3 | -11.370 |
| 2 | REF. 2 | 126.9 | 4365 | 2.7 | -11.557 |
| 3 | REF. 3 | 166.6 | 4353 | 3.1 | -11.642 |
| 4 | REF. 4 | 206.5 | 4357 | 3.2 | -11.648 |
| 5 | REF. 5 | 445.4 | 4346 | 31.9 | -11.587 |
| 6* | REF. 6 | 465.1 | 4347 | 35.1 | -11.700 |
| 7 | 1 | 545.7 | 1613 | 39.5 | -21.288 |
| 8 | 2 | 561.2 | 2459 | 45.6 | -20.431 |
| 9 | REF. 7 | 1052.5 | 4362 | 85.3 | -11.653 |
| 10 | REF. 8 | 1102.3 | 4359 | 89.0 | -11.751 |
| 11 | REF. 9 | 1162.0 | 4355 | 2.6 | -11.634 |
| 12 | REF. 10 | 1201.8 | 4370 | 2.5 | -11.624 |

GDC00870



Poste de travail

[illegible]

backump311006-1(D:)

Fichier Edition Affichage Favoris Outils


Précédente Recherche Dossier Historique

Adresse backump 311006-1 (D:)

| Nom | Type | Taille | Date | Modifié le |
|-----------------------------------|---------------------|--------|------------------|------------|
| <input type="checkbox"/> 191006 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 200106 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 200206 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 200306 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 200606 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 200706 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 200906 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 201006 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 201206 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 210306 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 210606 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 210706 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 210906 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 220206 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 220306 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 220606 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 220706 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 220906 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 220206 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 220306 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 230506 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 230606 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 230706 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 230806 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 231006 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 240306 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 240506 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 240706 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 240806 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 241006 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 250806 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 250906 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> 251006 | Dossier de fichiers | | 31/10/2006 12:33 | |
| <input type="checkbox"/> ISOVTECH | Dossier de fichiers | | 31/10/2006 12:33 | |

Sélectionnez un élément pour obtenir une description.

Capacité : 679 Mo
☒ LMSd : 679 Mo
☐ Libre : 0 octets



Voir aussi :
 Mes documents
 Favoris récents
 Poste de travail

COPIE CERTIFIÉE
CONFORME DES DOSSIERS
ET FICHILIAIRES ORIGINAUX

| Fichier Edition Affichage Paramètres Outils ? | | | | |
|---|-------------|--------|-------------|------------------|
| Précédente → Recherche Dossier Historique | | | | |
| 230706 | 230706 | | | |
| Nom | Type | Taille | Type | Modifié le |
| DATA_001.DFF | Fichier DFF | 8 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_001.DFF | Fichier DFF | 105 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_002.DFF | Fichier DFF | 8 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_002.DFF | Fichier DFF | 105 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_003.DFF | Fichier DFF | 8 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_003.DFF | Fichier DFF | 105 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_004.DFF | Fichier DFF | 9 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_004.DFF | Fichier DFF | 137 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_005.DFF | Fichier DFF | 9 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_005.DFF | Fichier DFF | 137 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_006.DFF | Fichier DFF | 9 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_006.DFF | Fichier DFF | 137 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_007.DFF | Fichier DFF | 10 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_007.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_008.DFF | Fichier DFF | 11 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_008.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_009.DFF | Fichier DFF | 11 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_009.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_010.DFF | Fichier DFF | 10 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_010.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_011.DFF | Fichier DFF | 11 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_011.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_012.DFF | Fichier DFF | 9 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_012.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_013.DFF | Fichier DFF | 9 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_013.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_014.DFF | Fichier DFF | 10 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_014.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |
| DATA_015.DFF | Fichier DFF | 409 Ko | Fichier DFF | 31/10/2006 12:30 |

Sélectionnez un élément pour obtenir une description.

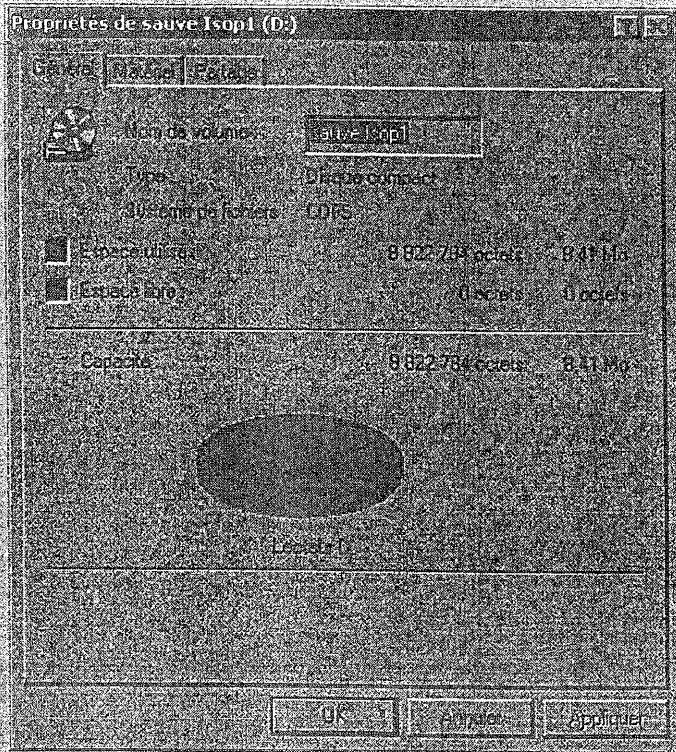
Voir aussi :
 Mes documents
 Réseaux réseau
 Poste de travail

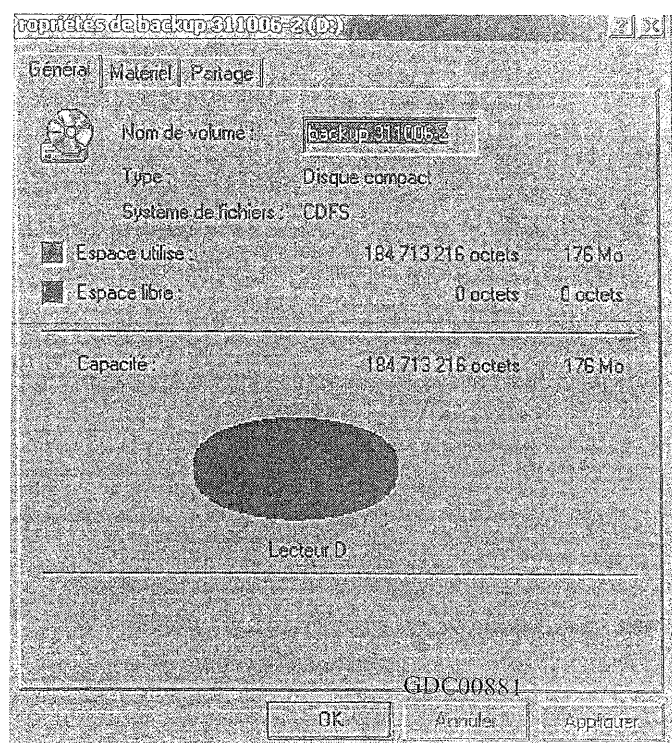
23 objets(s)

COPIE CERTIFIÉE
CONFORMÉ DES DOSSIER
ET FORMALITÉS ORIGINAUX

| 040806 | | | | Fichier Edition Affichage Favoris Outils 7 | | | | Rechercher Dossier Historique | | | | Fichier Edition Affichage Favoris Outils 7 | | | |
|---|-------------|--------|------------------|--|--|--|--|-------------------------------|--|--|--|--|--|--|--|
| Adi 6556 040806 | | | | | | | | | | | | | | | |
| 040806 | | | | | | | | | | | | | | | |
| Sélectionnez un élément pour obtenir une description. | | | | | | | | | | | | | | | |
| voir aussi : | | | | | | | | | | | | | | | |
| Mes documents | | | | | | | | | | | | | | | |
| Favoris Réseau | | | | | | | | | | | | | | | |
| Poste de travail | | | | | | | | | | | | | | | |
| Item | Type | Taille | Modifié | | | | | | | | | | | | |
| DATA_001.DPF | Fichier DPF | 6 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_001.DPF | Fichier DPF | 105 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_002.DPF | Fichier DPF | 8 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_002.DPF | Fichier DPF | 105 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_003.DPF | Fichier DPF | 8 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_003.DPF | Fichier DPF | 105 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_004.DPF | Fichier DPF | 8 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_004.DPF | Fichier DPF | 105 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_005.DPF | Fichier DPF | 8 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_005.DPF | Fichier DPF | 105 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_006.DPF | Fichier DPF | 9 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_006.DPF | Fichier DPF | 137 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_007.DPF | Fichier DPF | 9 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_007.DPF | Fichier DPF | 137 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_008.DPF | Fichier DPF | 9 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_008.DPF | Fichier DPF | 137 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_009.DPF | Fichier DPF | 10 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_009.DPF | Fichier DPF | 409 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_010.DPF | Fichier DPF | 12 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_010.DPF | Fichier DPF | 409 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_011.DPF | Fichier DPF | 12 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_011.DPF | Fichier DPF | 409 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_012.DPF | Fichier DPF | 9 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_012.DPF | Fichier DPF | 409 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_013.DPF | Fichier DPF | 9 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_013.DPF | Fichier DPF | 409 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_014.DPF | Fichier DPF | 9 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_014.DPF | Fichier DPF | 409 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_015.DPF | Fichier DPF | 9 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_015.DPF | Fichier DPF | 409 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_016.DPF | Fichier DPF | 10 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |
| DATA_016.DPF | Fichier DPF | 409 Ko | 31/10/2006 12:29 | | | | | | | | | | | | |

7





13 GDC00882

[illegible]

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

| 260606 | Fichier | Extension | Affichage | Favors | Outils | Recherche | Postes | Navigation | Modèle | Modèle |
|--------|--------------|-----------|-------------|------------------|--------|-----------|--------|------------|--------|--------|
| 260606 | DATA_001.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_001.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_002.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_002.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_003.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_003.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_004.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_004.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_005.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_005.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_006.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_006.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_007.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_007.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_008.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_008.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_009.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_009.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_010.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_010.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_011.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_011.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_012.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_012.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_013.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_013.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_014.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_014.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_015.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_015.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_016.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_016.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_017.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_017.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |
| | DATA_018.DPF | 8 Ko | Fichier 000 | 31/10/2006 12:44 | | | | | | |
| | DATA_018.DPF | 105 Ko | Fichier DPF | 31/10/2006 12:44 | | | | | | |

Sélectionner un élément pour obtenir une description.

Voir aussi :

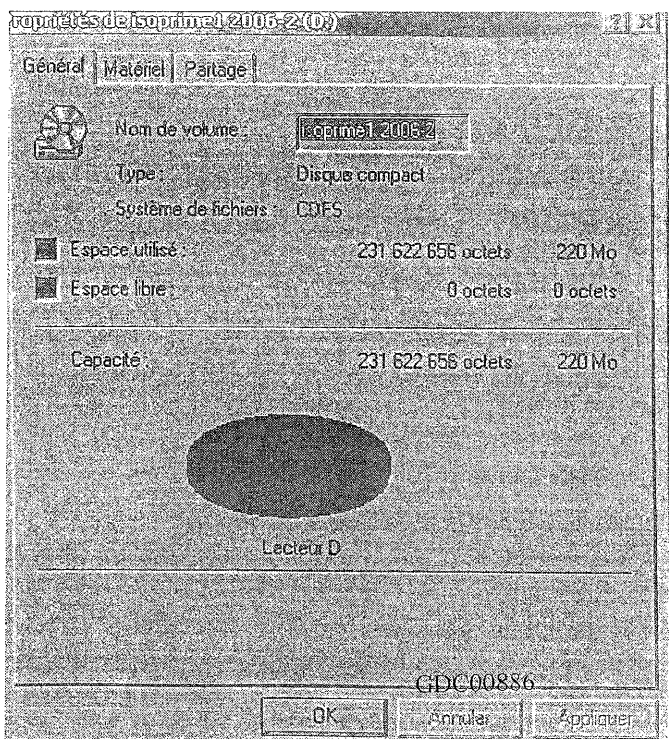
Mes documents

Favoris récents

Poste de travail

16

GDC00885



Fichier Edition Affichage Fenêtre Outil ?

Précédente **Rechercher** **Dossier** **Historique** **Quitter** **Aide**

Adresse: 310706

| Nom | Taille | Type | Date |
|------------------|--------|-------------|------------------|
| [] DATA_001.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_001.DPF | 105 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_002.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_002.DPF | 105 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_003.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_003.DPF | 105 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_004.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_004.DPF | 105 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_005.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_005.DPF | 105 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_006.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_006.DPF | 105 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_007.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_007.DPF | 108 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_008.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_008.DPF | 108 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_009.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_009.DPF | 108 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_010.000 | 8 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_010.DPF | 99 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_011.000 | 9 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_011.DPF | 137 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_012.000 | 9 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_012.DPF | 137 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_013.000 | 9 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_013.DPF | 137 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_014.000 | 9 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_014.DPF | 137 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_015.000 | 9 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_015.DPF | 137 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_016.000 | 9 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_016.DPF | 137 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_017.000 | 10 Ko | Fichier 000 | 30/01/2007 20:03 |
| [x] DATA_017.DPF | 409 Ko | Fichier DPF | 30/01/2007 20:03 |
| [] DATA_018.000 | 10 Ko | Fichier 000 | 30/01/2007 20:03 |

Sélectionnez un élément pour obtenir une description.

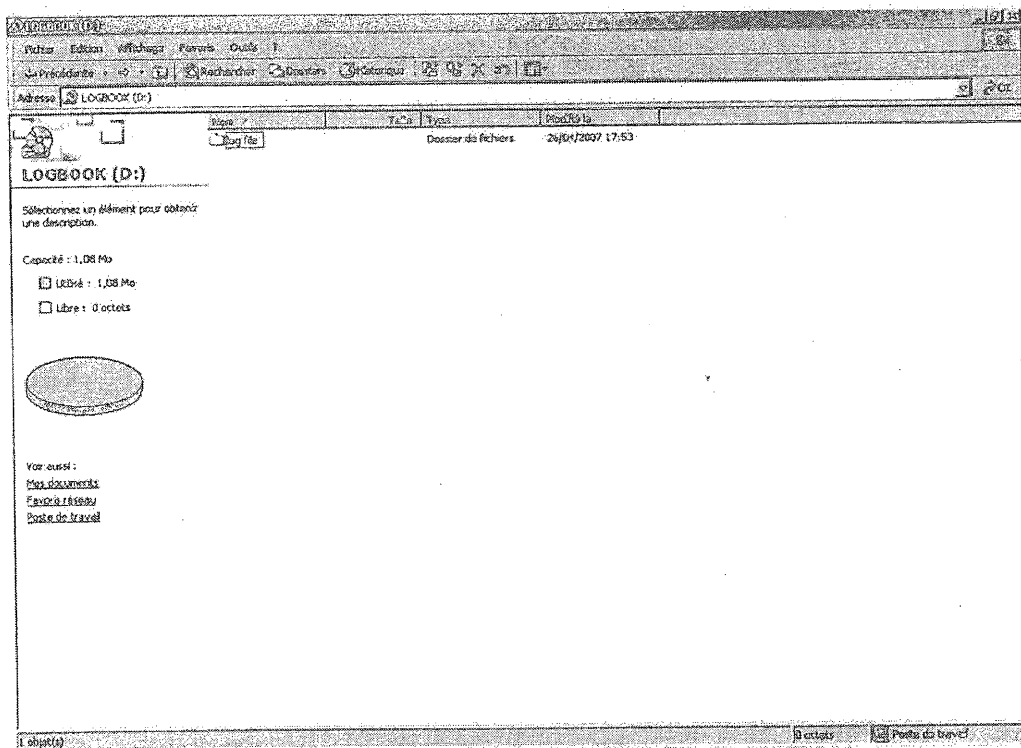
Voir aussi :

- Mes documents
- Cartographie réseau
- Page de travail

310706

852 objet(s)

Reste à travailler



COPIE CLAIR
 COPIE DES DONNÉES
 ET FORMAIRES ORIGINAUX

20

GDC00889

Microsoft Office Word 2003

Menu: Fichier, Edition, Affichage, Favoris, Outils

Barre d'outils: Recherche, Descripteur, Messages

Adresse: log file

| Nom | Taille | Type | Date |
|------------|--------|-----------------|------------------|
| Excel | 88 Ko | Texte seulement | 26/04/2007 17:52 |
| loadp | 74 Ko | Texte seulement | 26/04/2007 17:52 |
| MessageLog | 871 Ko | Texte seulement | 26/04/2007 17:52 |

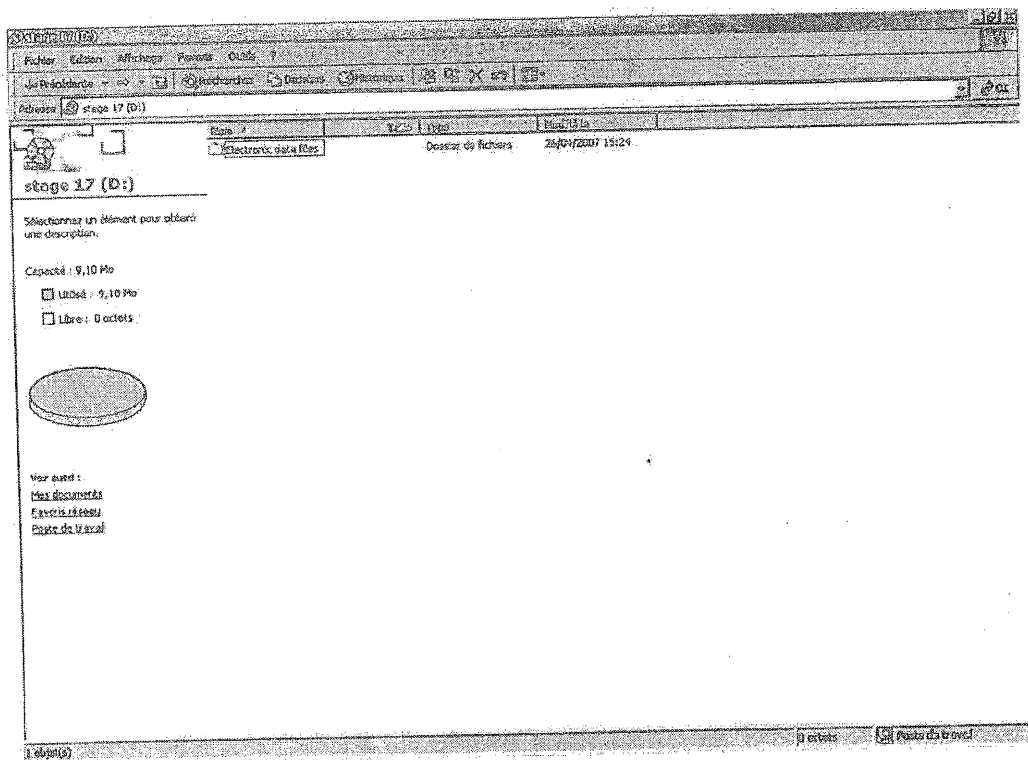
log file

Sélectionnez un élément pour obtenir une description.

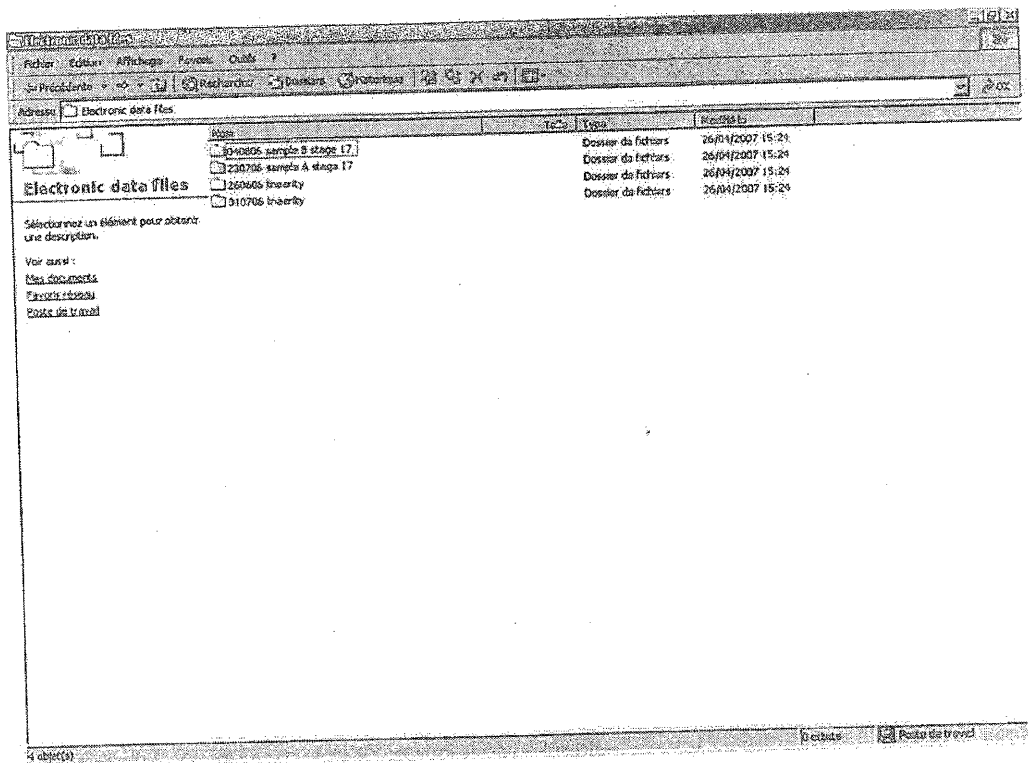
Voir aussi :

- Des documents
- Favorites
- Page de travail

9,00 Mo (s) 11,00 Mo Poste de travail



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMILAIRES ORIGINAUX



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

23

| 230706 sample A stage 17 | | | |
|--------------------------|--------|-------------|------------------|
| Nom | Taille | Type | Modifié |
| DATA_001.DPF | 8 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_001.DPF | 105 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_002.DPF | 8 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_002.DPF | 105 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_003.DPF | 8 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_003.DPF | 105 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_004.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_004.DPF | 127 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_005.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_005.DPF | 127 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_006.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_006.DPF | 127 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_007.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_007.DPF | 409 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_008.DPF | 11 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_008.DPF | 409 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_009.DPF | 11 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_009.DPF | 409 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_010.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_010.DPF | 409 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_011.DPF | 11 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_011.DPF | 409 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_012.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_012.DPF | 409 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_013.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_013.DPF | 409 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_014.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 |
| DATA_014.DPF | 409 Ko | Fichier DPF | 26/04/2007 15:24 |

COPIE CERTIFIÉE
CONFORME AUX DONNÉES
ET FORMULAIRES ORIGINAUX

24

GDC00893

| Fichiers | | | | 10/2/2 |
|--|--------|-------------|------------------|--------|
| Fichier Edition Affichage Format Outils | | | | 80 |
| Préférences Rechercher Paramètres Historique | | | | |
| 040806 sample B stage 17 | | | | 05 |
| Item | Taille | Type | Date | |
| DATA_001.DPF | 8 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_002.DPF | 105 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_003.DPF | 8 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_004.DPF | 105 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_005.DPF | 8 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_006.DPF | 105 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_007.DPF | 8 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_008.DPF | 105 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_009.DPF | 8 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_010.DPF | 105 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_011.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_012.DPF | 137 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_013.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_014.DPF | 137 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_015.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_016.DPF | 137 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_017.DPF | 9 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_018.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_019.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_020.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_021.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_022.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_023.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_024.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_025.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_026.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_027.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_028.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_029.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_030.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_031.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_032.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_033.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_034.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_035.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_036.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_037.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_038.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_039.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_040.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_041.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_042.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_043.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_044.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_045.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_046.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_047.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_048.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_049.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |
| DATA_050.DPF | 10 Ko | Fichier DPF | 26/04/2007 15:24 | |

GDC00894

260606 linearity

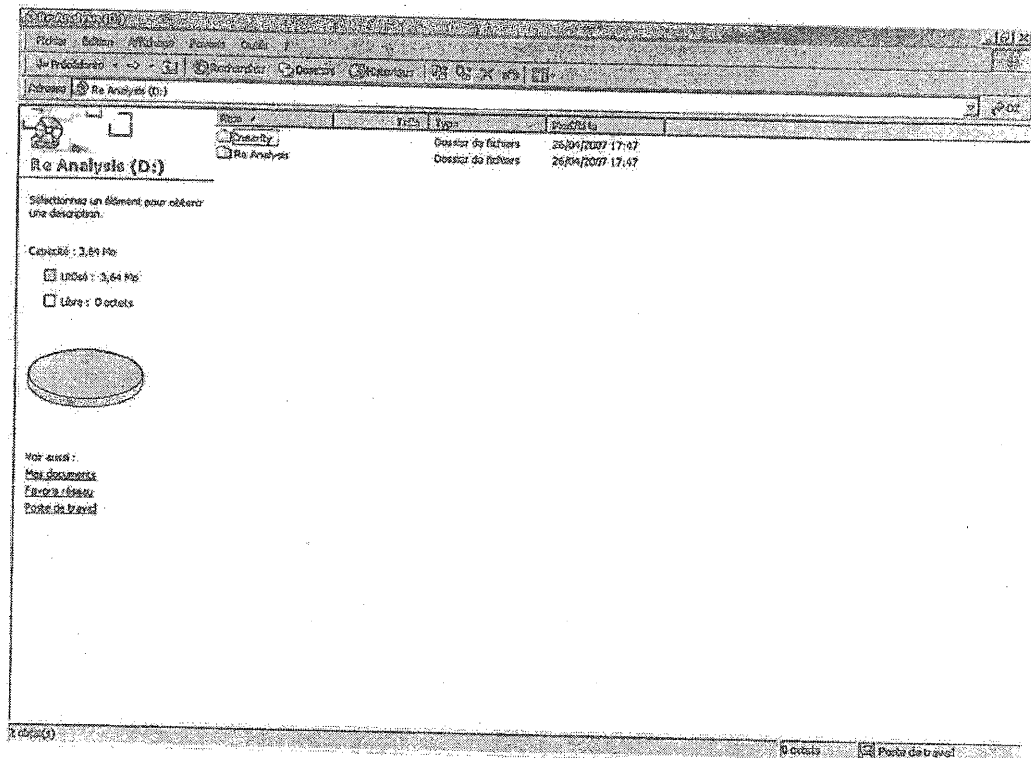
260606 linearity

Sélectionner un élément pour obtenir une description.

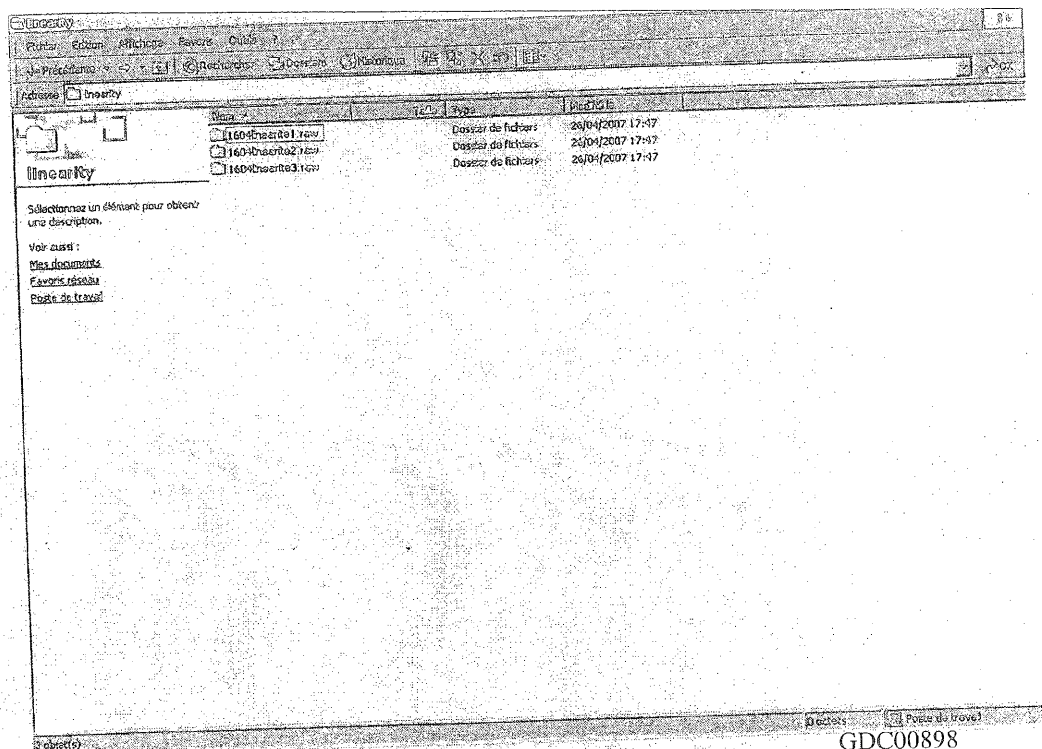
Voir aussi:
Mes documents
Favoris réseau
Porte de travail

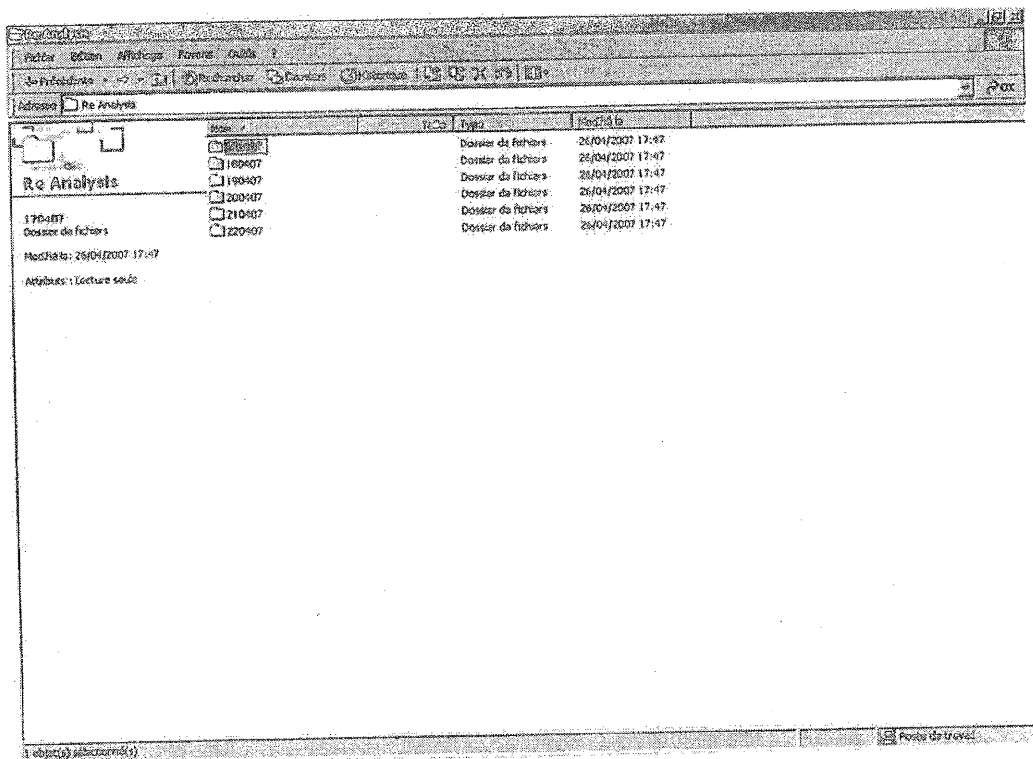
| Prén | Taille | Type | Modifié |
|--|--------|-------------|------------------|
| <input checked="" type="checkbox"/> DATA_008.000 | 8 Ko | Fichier ODD | 26/04/2007 15:24 |
| <input checked="" type="checkbox"/> DATA_008.DPF | 108 Ko | Fichier DPF | 26/04/2007 15:24 |
| <input checked="" type="checkbox"/> DATA_009.000 | 8 Ko | Fichier ODD | 26/04/2007 15:24 |
| <input checked="" type="checkbox"/> DATA_009.DPF | 108 Ko | Fichier DPF | 26/04/2007 15:24 |
| <input checked="" type="checkbox"/> DATA_010.000 | 8 Ko | Fichier ODD | 26/04/2007 15:24 |
| <input checked="" type="checkbox"/> DATA_010.DPF | 108 Ko | Fichier DPF | 26/04/2007 15:24 |

0 objet(s)316 KoPorte de travail





COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMATAIRES ORIGINAUX





COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMELAIRES ORIGINAUX


| | | | | | |
|--|--|--|--|--|--|
| VIR00100 | | | | 15/11/2007 | |
| Fichier Edition Affichage Fenêtre Outil ? | | | | | |
| Précédente Recherche Dossier Fichiers | | | | | |
| Adresse 170407 | | | | | |
| <div>   </div> <div>170407</div> <div> Sélectionnez un élément pour obtenir une description. Voir aussi : Mes documents Favoris réseau Poste de travail </div> | | <div> <div>1704425F1.raw</div> <div>1704425F2.raw</div> <div>1704425F3.raw</div> <div>1704801F1.raw</div> <div>1704801F2.raw</div> <div>1704801F3.raw</div> <div>17048021.raw</div> <div>1704MxColAcetate01.raw</div> <div>1704MxColAcetate02.raw</div> <div>1704MxColRM501.raw</div> <div>1704MxColRM502.raw</div> <div>1704MxColRM503.raw</div> <div>1704MxColRM504.raw</div> <div>1704stabil01.raw</div> <div>1704stabil02.raw</div> <div>1704stabil03.raw</div> </div> | <div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> <div>Dossier de fichiers</div> </div> | <div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> <div>26/04/2007 17:47</div> </div> | |

GDC00900

GDC00900

| 190407 | | | |
|---|---------------------|------------|-------|
| Fichier Edition Affichage Favoris Outils | | | |
| Préférences Rechercher Diagnostics Paramètres | | | |
| Adresses 190407 | | | |
| Map | Type | Date/PS | |
| <input type="checkbox"/> 1904426F1.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904426F2.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904426F3.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904426F1.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904426F2.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904426F3.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904426F3-2.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904651F1.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904651F2.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904651F3.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904652F1.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904652F2.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 1904652F3.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 190440c6aAcetate01.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 190440c6aAcetate02.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 190440c6aIRMS01.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 190440c6aIRMS02.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 190440c6aIRMS03.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 190440c6a1.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 190440c6a2.raw | Dossier de fichiers | 26/04/2007 | 17:47 |
| <input type="checkbox"/> 190440c6a3.raw | Dossier de fichiers | 26/04/2007 | 17:47 |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET Fournitures ORIGINAUX

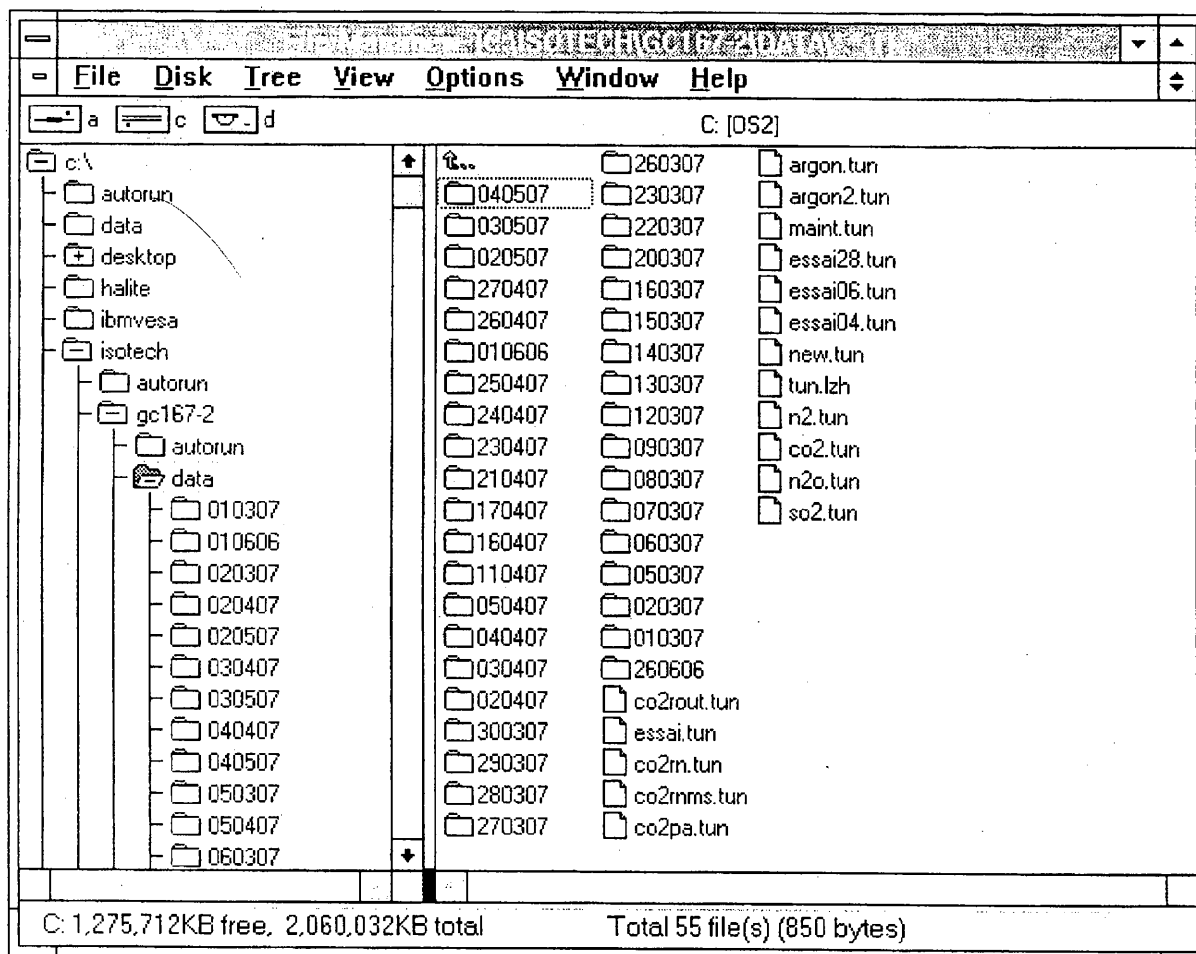
| 200407 | | | |
|---|---------------------------------------|---------------------|------------------|
| Fichier Edition Affichage Favoris Outils | | | |
| Présentation Rechercher Documents Historique | | | |
| Adresse 200407 | | | |
| | Nom | Type | Modifié le |
|  200407 Sélectionnez un élément pour obtenir une description. Voir aussi : 20040701 20040702 20040703 20040704 | <input type="checkbox"/> 20040701.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040702.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040703.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040704.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040705.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040706.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040707.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040708.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040709.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040710.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040711.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040712.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040713.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040714.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040715.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040716.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040717.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040718.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040719.raw | Dossier de fichiers | 26/04/2007 17:47 |
| | <input type="checkbox"/> 20040720.raw | Dossier de fichiers | 26/04/2007 17:47 |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

| 210407 | | | |
|---|-------------|---------------------|------------------|
| Fichier Edition Affichage Paramètres Outils | | | |
| Rechercher Contenu Historique | | | |
| Adresse 210407 | | | |
| Icon | Nom | Type | Modifié le |
| | 210407 | | |
| | 2104071.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104072.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104073.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104074.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104075.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104076.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104077.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104078.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104079.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104080.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104081.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104082.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104083.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104084.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104085.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104086.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104087.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104088.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104089.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104090.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104091.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104092.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104093.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104094.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104095.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104096.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104097.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104098.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104099.ram | Dossier de fichiers | 26/04/2007 17:47 |
| | 2104100.ram | Dossier de fichiers | 26/04/2007 17:47 |
| Sélectionnez un élément pour obtenir une description. | | | |
| Voir aussi : | | | |
| Mes documents | | | |
| Fichiers récents | | | |
| Poste de travail | | | |
| 20 objets | | | |
| Détails | | | |
| Poste de travail | | | |

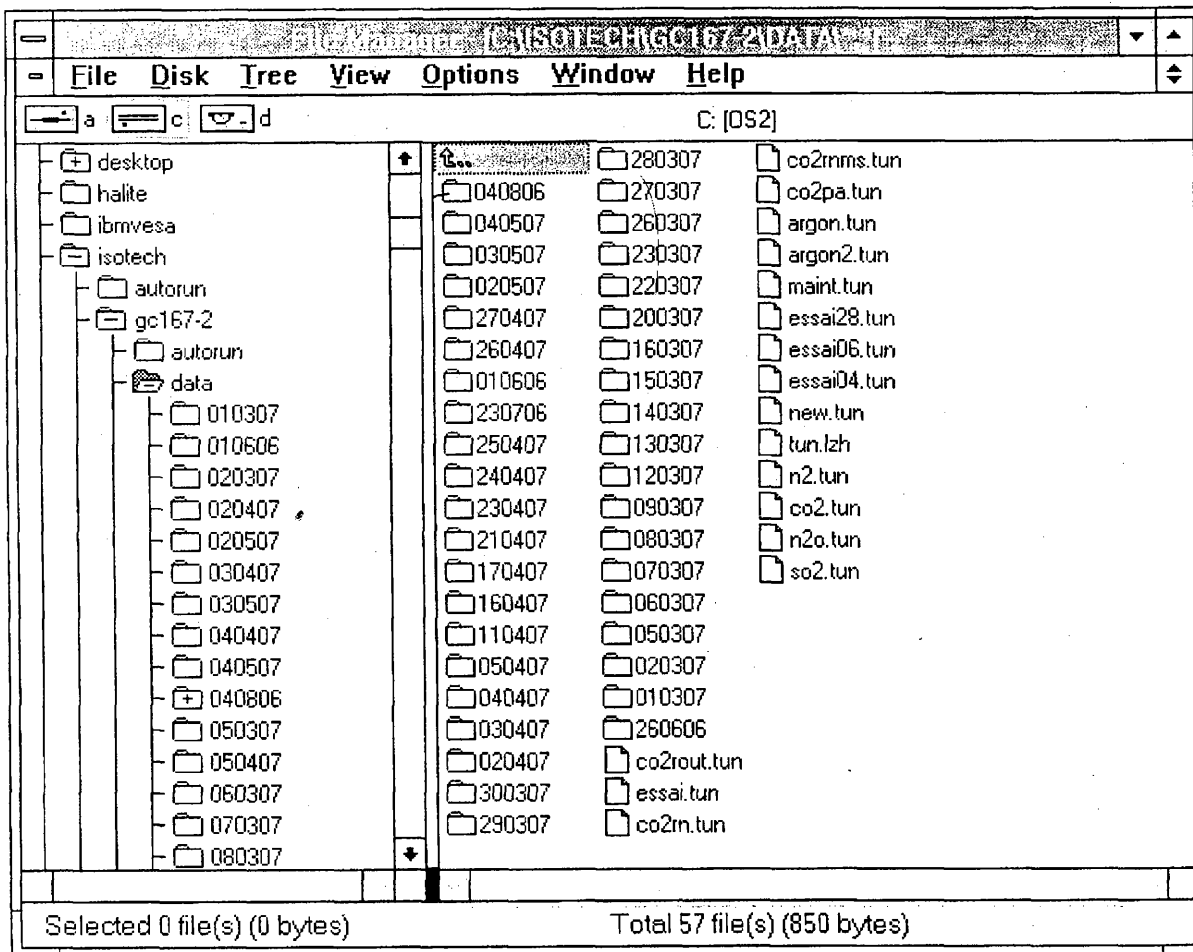
COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

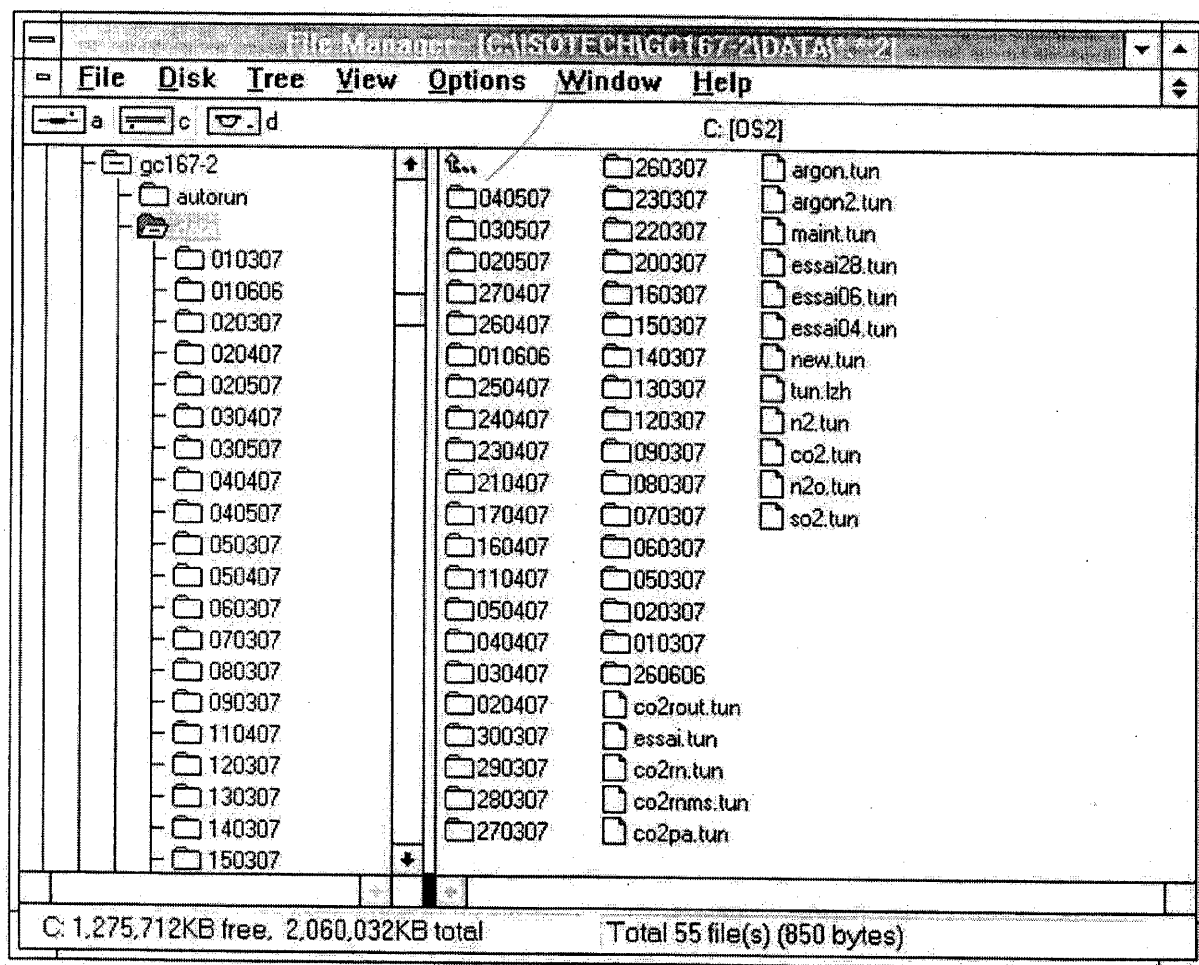


COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMAIRES ORIGINAUX

37



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

Auto

Data Processing Results

Data File Name : DATA_007
 Folder : 230706
 Sample Name : Mix cal Acetate 001A-100ng inj
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 10:53:36 Date : 23/07/06
 Current Time : 17:07:21 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.609E-8 | 1.1781E-2 | 4.2521E-3 |
| 182.6 | 8.616E-8 | 1.1781E-2 | 4.2522E-3 |
| 242.6 | 8.618E-8 | 1.1781E-2 | 4.2523E-3 |
| 2423.4 | 8.509E-8 | 1.1781E-2 | 4.2519E-3 |
| 2483.5 | 8.465E-8 | 1.1781E-2 | 4.2516E-3 |
| 2533.4 | 8.528E-8 | 1.1780E-2 | 4.2520E-3 |

Std Dev Of Fit 1.7125E-7 1.7134E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|
| | 866.6 | 4.79E-9 | 2.0679E-8 | 1.1821E-2 | 4.1654E-3 | -30.29 | -68.47 | -39.32 |
| | 1229.8 | 4.79E-9 | 3.8612E-8 | 1.1938E-2 | 4.1669E-3 | -20.05 | -66.68 | -38.97 |
| | 1302.2 | 3.63E-9 | 2.8817E-8 | 1.1782E-2 | 4.1665E-3 | -33.74 | -66.53 | -39.03 |
| | 1473.8 | 3.17E-9 | 3.4144E-8 | 1.1975E-2 | 4.1670E-3 | -16.83 | -66.28 | -38.96 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

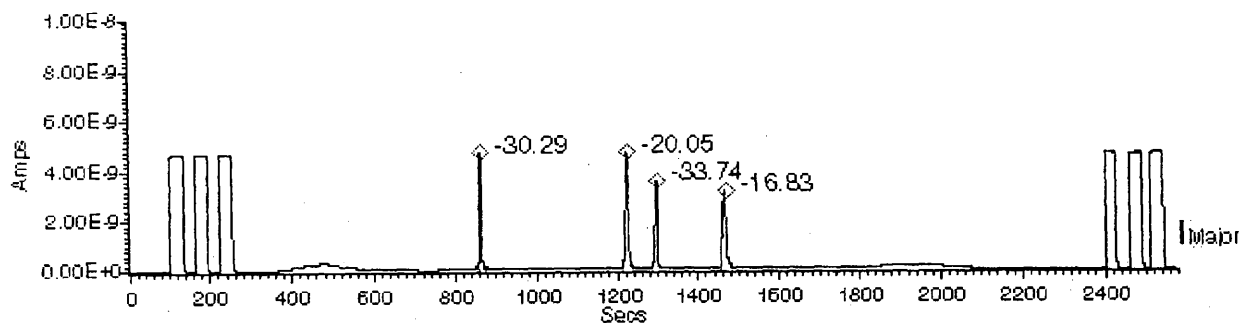
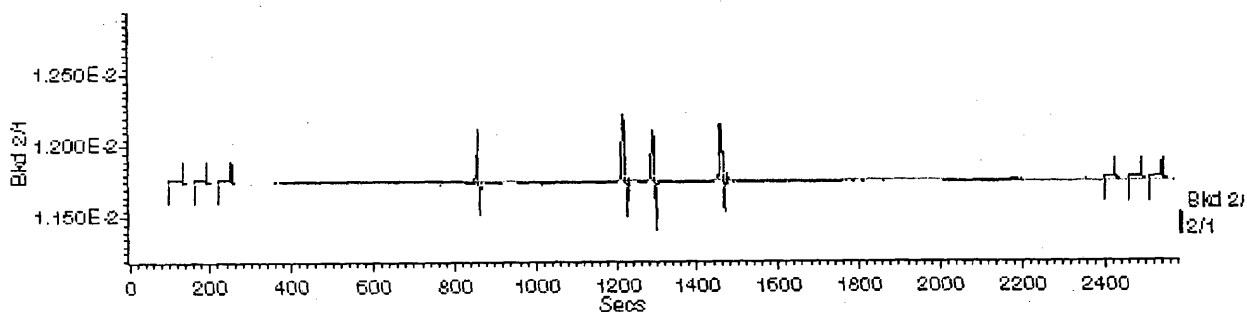
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_007 Folder : 230706
 Date : 23/07/06 Time : 10:53:36
 Comment : Mix cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

/./

GDC00910

manual

Data Processing Results

Data File Name : DATA_007
Folder : 230706
Sample Name : Mix cal Acetate 001A-100ng inj
Sample ID :
Sample Position : 2
Injection Size : 0.0000
Sample Type : Sam
Method : M-AN-41
Batch Name :
RunTime User : micromass
Acquisition Time : 10:53:36 Date : 23/07/06
Current Time : 16:17:16 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.609E-8 | 1.1781E-2 | 4.2521E-3 |
| 182.6 | 8.616E-8 | 1.1781E-2 | 4.2522E-3 |
| 242.6 | 8.618E-8 | 1.1781E-2 | 4.2523E-3 |
| 2423.4 | 8.509E-8 | 1.1781E-2 | 4.2519E-3 |
| 2483.5 | 8.465E-8 | 1.1781E-2 | 4.2516E-3 |
| 2533.4 | 8.528E-8 | 1.1780E-2 | 4.2520E-3 |

Std Dev Of Fit 1.7125E-7 1.7134E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 866.6 | 4.79E-9 | 2.0679E-8 | 1.1822E-2 | 4.1654E-3 | -30.20 | -69.24 | -39.32 | 24 |
| | 1229.8 | 4.79E-9 | 3.8612E-8 | 1.1938E-2 | 4.1669E-3 | -20.02 | -66.90 | -38.97 | 24 |
| | 1302.2 | 3.63E-9 | 2.8817E-8 | 1.1782E-2 | 4.1665E-3 | -33.71 | -66.68 | -39.03 | 24 |
| | 1473.8 | 3.17E-9 | 3.2851E-8 | 1.1976E-2 | 4.1667E-3 | -16.72 | -66.32 | -39.03 | 24 |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

42

GDC00911

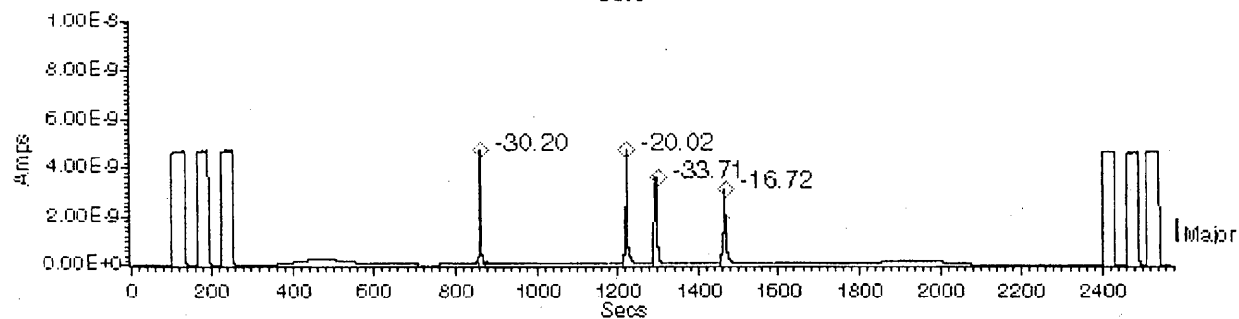
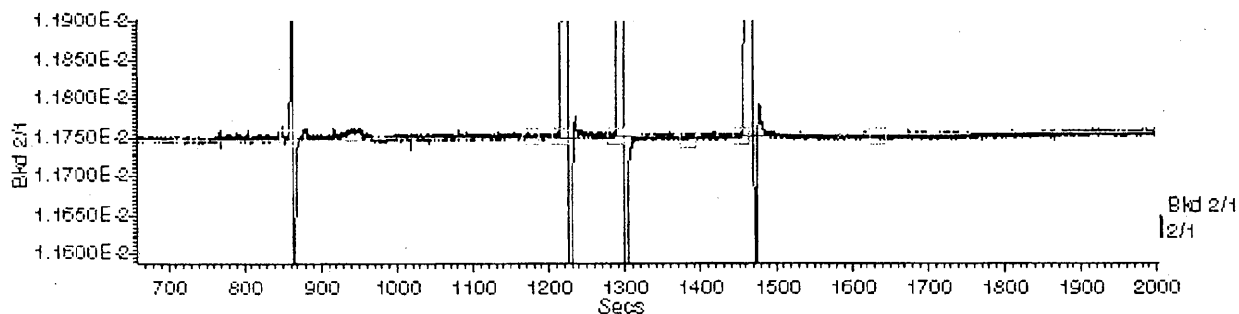
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_007 Folder : 230706
 Date : 23/07/06 Time : 10:53:36
 Comment : Mix cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

◀ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

43

GDC00912

Zero

Data Processing Results

Data File Name : DATA_007
 Folder : 230706
 Sample Name : Mix cal Acetate 001A-100ng inj
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 10:53:36 Date : 23/07/06
 Current Time : 17:09:58 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.609E-8 | 1.1781E-2 | 4.2521E-3 |
| 182.6 | 8.616E-8 | 1.1781E-2 | 4.2522E-3 |
| 242.6 | 8.618E-8 | 1.1781E-2 | 4.2523E-3 |
| 2423.4 | 8.509E-8 | 1.1781E-2 | 4.2519E-3 |
| 2483.5 | 8.465E-8 | 1.1781E-2 | 4.2516E-3 |
| 2533.4 | 8.528E-8 | 1.1780E-2 | 4.2520E-3 |

Std Dev Of Fit 1.7125E-7 1.7134E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 866.6 | 4.79E-9 | 2.0679E-8 | 1.1822E-2 | 4.1655E-3 | -30.19 | -39.30 > |
| 1229.8 | 4.79E-9 | 3.8612E-8 | 1.1931E-2 | 4.1662E-3 | -20.66 | -39.14<> |
| 1302.2 | 3.63E-9 | 2.8817E-8 | 1.1790E-2 | 4.1656E-3 | -33.01 | -39.25<> |
| 1473.8 | 3.17E-9 | 3.4144E-8 | 1.1961E-2 | 4.1649E-3 | -18.01 | -39.43< |

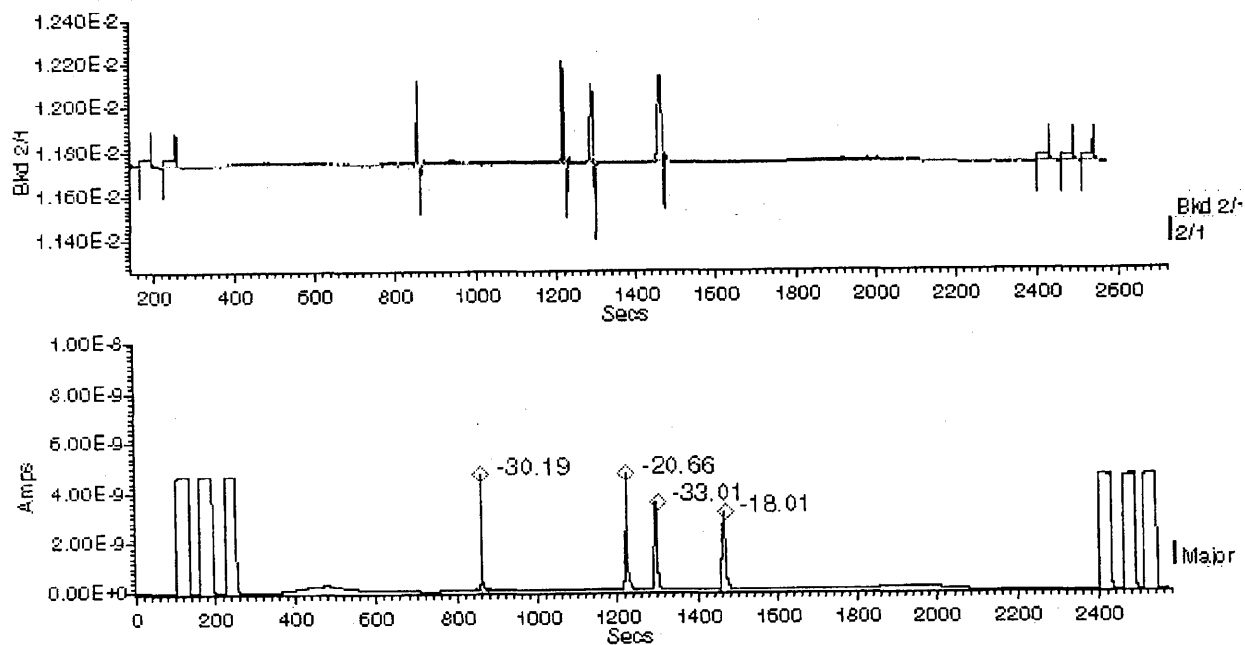
COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_007 Folder : 230706
 Date : 23/07/06 Time : 10:53:36
 Comment : Mix cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

45

Data Processing Results

Data File Name : DATA_008
 Folder : 230706
 Sample Name : Blu 1 pool 4 F3/45ul inj 2ul
 Sample ID :
 Sample Position : 3
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 11:40:11 Date : 23/07/06
 Current Time : 18:26:52 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.660E-8 | 1.1780E-2 | 4.2525E-3 |
| 182.6 | 8.668E-8 | 1.1781E-2 | 4.2527E-3 |
| 242.6 | 8.673E-8 | 1.1781E-2 | 4.2531E-3 |
| 2423.5 | 8.560E-8 | 1.1783E-2 | 4.2534E-3 |
| 2483.5 | 8.558E-8 | 1.1782E-2 | 4.2529E-3 |
| 2533.5 | 8.621E-8 | 1.1782E-2 | 4.2533E-3 |

Std Dev Of Fit 5.9183E-7 2.6505E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 798.5 | 2.00E-8 | 1.1894E-7 | 1.1613E-2 | 4.4644E-3 | -50.88 | -57.08 | 29.56 | 9 |
| | 867.4 | 6.17E-9 | 2.4902E-8 | 1.1841E-2 | 4.1918E-3 | -28.78 | -57.69 | -33.41 | 11 |
| | 1244.7 | 2.49E-9 | 2.1148E-8 | 1.1835E-2 | 4.1951E-3 | -29.40 | -59.42 | -32.66 | 18 |
| | 1306.2 | 7.10E-9 | 6.5412E-8 | 1.1850E-2 | 4.1696E-3 | -27.84 | -59.54 | -38.55 | 19 |
| | 1336.6 | 2.32E-9 | 1.7757E-8 | 1.1836E-2 | 4.1791E-3 | -29.16 | -59.63 | -36.36 | 19 |
| | 1375.6 | 1.42E-9 | 1.2239E-8 | 1.1837E-2 | 4.1854E-3 | -29.15 | -59.66 | -34.91 | 19 |
| | 1651.5 | 3.59E-9 | 3.9165E-8 | 1.1858E-2 | 4.1692E-3 | -27.19 | -59.37 | -38.67 | 20 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

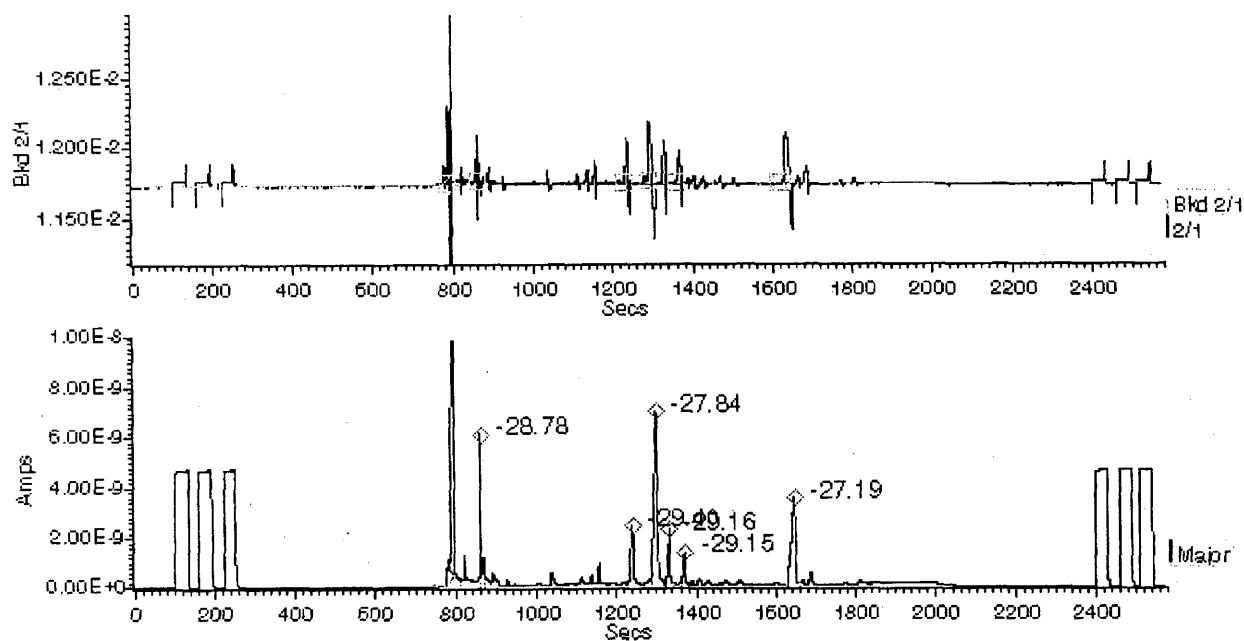
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename: DATA_008 Folder: 230706
 Date: 23/07/06 Time: 11:40:11
 Comment: Blu 1 pool 4 F3/45ul inj 2ul:
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

47

GDC00916

Data Processing Results

Auto

Data File Name : DATA_008
 Folder : 230706
 Sample Name : Blu 1 pool 4 F3/45ul inj 2ul
 Sample ID :
 Sample Position : 3
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 11:40:11 Date : 23/07/06
 Current Time : 17:18:18 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.660E-8 | 1.1780E-2 | 4.2525E-3 |
| 182.6 | 8.668E-8 | 1.1781E-2 | 4.2527E-3 |
| 242.6 | 8.673E-8 | 1.1781E-2 | 4.2531E-3 |
| 2423.5 | 8.560E-8 | 1.1783E-2 | 4.2534E-3 |
| 2483.5 | 8.558E-8 | 1.1782E-2 | 4.2529E-3 |
| 2533.5 | 8.621E-8 | 1.1782E-2 | 4.2533E-3 |

Std Dev Of Fit 5.9183E-7 2.6505E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|
| 798.5 | 2.00E-8 | 1.2123E-7 | 1.1611E-2 | 4.4640E-3 | -51.06 | -46.73 | 29.47 |
| 867.4 | 6.17E-9 | 2.4902E-8 | 1.1834E-2 | 4.1918E-3 | -29.43 | -47.23 | -33.41 |
| 1244.7 | 2.49E-9 | 2.1148E-8 | 1.1812E-2 | 4.1951E-3 | -31.41 | -51.69 | -32.66 |
| 1306.2 | 7.10E-9 | 6.5412E-8 | 1.1845E-2 | 4.1696E-3 | -28.31 | -52.66 | -38.55 |
| 1336.6 | 2.32E-9 | 1.8245E-8 | 1.1820E-2 | 4.1806E-3 | -30.56 | -53.24 | -36.02 |
| 1375.6 | 1.42E-9 | 1.2239E-8 | 1.1816E-2 | 4.1854E-3 | -30.95 | -53.93 | -34.90 |
| 1651.5 | 3.59E-9 | 3.9165E-8 | 1.1859E-2 | 4.1692E-3 | -27.12 | -59.95 | -38.67 |

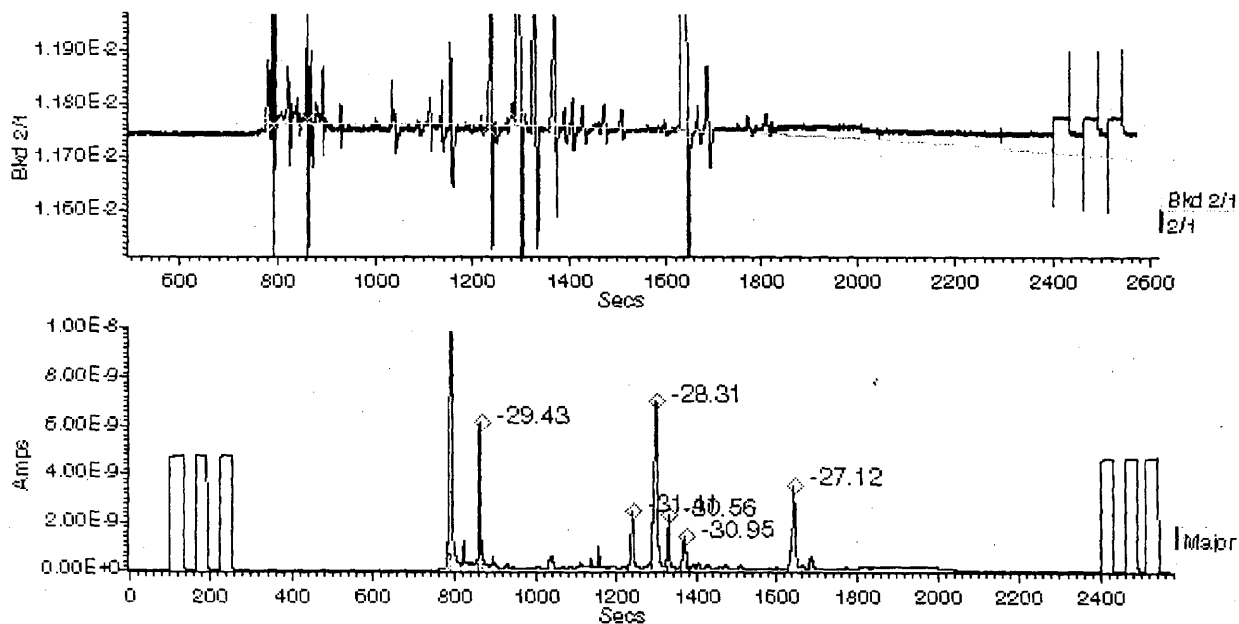
COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

48

Data Filename : DATA_008 Folder : 230706
 Date : 23/07/06 Time : 11:40:11
 Comment : Blu 1 pool 4 F3/45ul inj 2ul :
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

49

Zero

Data Processing Results

Data File Name : DATA_008
 Folder : 230706
 Sample Name : Blu 1 pool 4 F3/45ul inj 2ul
 Sample ID :
 Sample Position : 3
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 11:40:11 Date : 23/07/06
 Current Time : 17:13:39 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.660E-8 | 1.1780E-2 | 4.2525E-3 |
| 182.6 | 8.668E-8 | 1.1781E-2 | 4.2527E-3 |
| 242.6 | 8.673E-8 | 1.1781E-2 | 4.2531E-3 |
| 2423.5 | 8.560E-8 | 1.1783E-2 | 4.2534E-3 |
| 2483.5 | 8.558E-8 | 1.1782E-2 | 4.2529E-3 |
| 2533.5 | 8.621E-8 | 1.1782E-2 | 4.2533E-3 |

Std Dev Of Fit 5.9183E-7 2.6505E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 798.5 | 2.00E-8 | 1.2123E-7 | 1.1621E-2 | 4.4427E-3 | -50.06 | 24.55 > |
| 867.4 | 6.17E-9 | 2.4902E-8 | 1.1844E-2 | 4.1650E-3 | -28.36 | -39.60<> |
| 1244.7 | 2.49E-9 | 2.1148E-8 | 1.1846E-2 | 4.1674E-3 | -28.23 | -39.06<> |
| 1306.2 | 7.10E-9 | 6.5412E-8 | 1.1853E-2 | 4.1644E-3 | -27.57 | -39.77<> |
| 1336.6 | 2.32E-9 | 1.8245E-8 | 1.1844E-2 | 4.1671E-3 | -28.39 | -39.13<> |
| 1375.6 | 1.42E-9 | 1.2239E-8 | 1.1850E-2 | 4.1682E-3 | -27.89 | -38.89<> |
| 1651.5 | 3.59E-9 | 3.9165E-8 | 1.1863E-2 | 4.1656E-3 | -26.75 | -39.49< |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

50

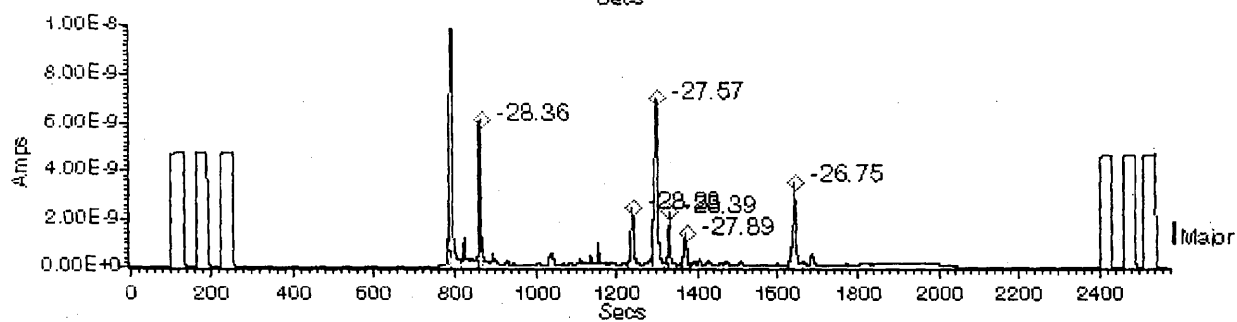
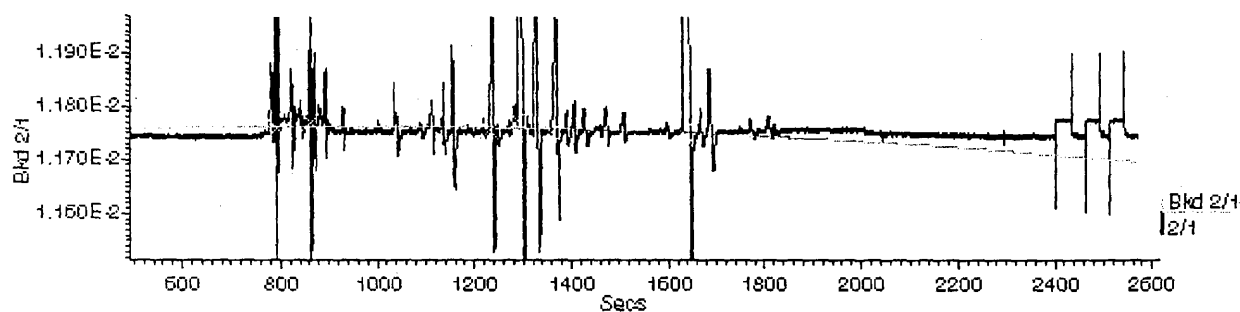
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_008 Folder : 230706
 Date : 23/07/06 Time : 11:40:11
 Comment : Blu 1 pool 4 F3/45ul inj 2ul:
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

manual.

Data File Name : DATA_009
 Folder : 230706
 Sample Name : 178/07 995474 F3/45ul inj 2ul
 Sample ID :
 Sample Position : 4
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 12:24:59 Date : 23/07/06
 Current Time : 17:41:33 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.494E-8 | 1.1782E-2 | 4.2529E-3 |
| 182.6 | 8.494E-8 | 1.1782E-2 | 4.2530E-3 |
| 242.6 | 8.494E-8 | 1.1781E-2 | 4.2527E-3 |
| 2423.5 | 8.443E-8 | 1.1780E-2 | 4.2518E-3 |
| 2483.5 | 8.453E-8 | 1.1779E-2 | 4.2517E-3 |
| 2533.5 | 8.566E-8 | 1.1779E-2 | 4.2520E-3 |

Std Dev Of Fit 5.6232E-7 1.7469E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 795.1 | 5.07E-9 | 2.5669E-8 | 1.1868E-2 | 4.2100E-3 | -26.50 | -48.53 | -29.16 | 5 |
| | 827.1 | 2.21E-9 | 1.0385E-8 | 1.1866E-2 | 4.2270E-3 | -26.82 | -48.83 | -25.22 | 6 |
| | 867.4 | 6.75E-9 | 2.9980E-8 | 1.1820E-2 | 4.1853E-3 | -30.57 | -49.11 | -34.83 | 7 |
| | 1304.7 | 5.53E-9 | 4.6423E-8 | 1.1835E-2 | 4.1673E-3 | -29.08 | -51.43 | -38.95 | 11 |
| | 1337.2 | 2.59E-9 | 2.1754E-8 | 1.1794E-2 | 4.1701E-3 | -32.63 | -51.54 | -38.30 | 12 |
| | 1652.0 | 3.25E-9 | 3.7635E-8 | 1.1864E-2 | 4.1682E-3 | -26.45 | -51.84 | -38.72 | 12 |

COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

52

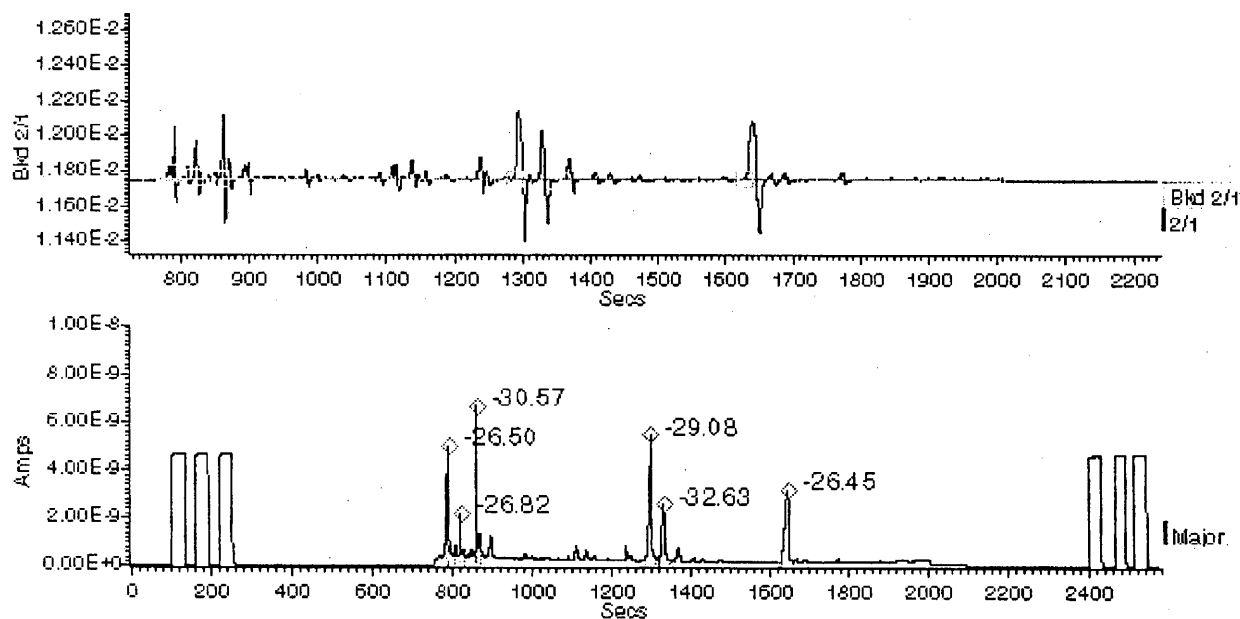
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_009 Folder : 230706
 Date : 23/07/06 Time : 12:24:59
 Comment : 178/07 995474 F3/45ul inj 2ul:
 Parameters Automatic DP Params

√ Data Processing Main Graph

Graph Cursor Lines Window



COPIL CERTIFIÉ
 CONFORME DES DONNÉES
 ET FORMULAIRES D'ANALYSE

Zero

Data Processing Results

Data File Name : DATA_009
 Folder : 230706
 Sample Name : 178/07 995474 F3/45ul inj 2ul
 Sample ID :
 Sample Position : 4
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 12:24:59 Date : 23/07/06
 Current Time : 17:43:17 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.494E-8 | 1.1782E-2 | 4.2529E-3 |
| 182.6 | 8.494E-8 | 1.1782E-2 | 4.2530E-3 |
| 242.6 | 8.494E-8 | 1.1781E-2 | 4.2527E-3 |
| 2423.5 | 8.443E-8 | 1.1780E-2 | 4.2518E-3 |
| 2483.5 | 8.453E-8 | 1.1779E-2 | 4.2517E-3 |
| 2533.5 | 8.566E-8 | 1.1779E-2 | 4.2520E-3 |

Std Dev Of Fit 5.6232E-7 1.7469E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 795.1 | 5.07E-9 | 2.5669E-8 | 1.1867E-2 | 4.1671E-3 | -26.29 | -39.06 > |
| 827.1 | 2.21E-9 | 1.0385E-8 | 1.1864E-2 | 4.1684E-3 | -26.52 | -38.74<> |
| 867.4 | 6.75E-9 | 2.9980E-8 | 1.1822E-2 | 4.1654E-3 | -30.22 | -39.44<> |
| 1304.7 | 5.53E-9 | 4.9528E-8 | 1.1836E-2 | 4.1648E-3 | -28.98 | -39.52<> |
| 1337.2 | 2.59E-9 | 2.3118E-8 | 1.1803E-2 | 4.1658E-3 | -31.85 | -39.28<> |
| 1652.0 | 3.25E-9 | 3.7635E-8 | 1.1860E-2 | 4.1661E-3 | -26.83 | -39.19< |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

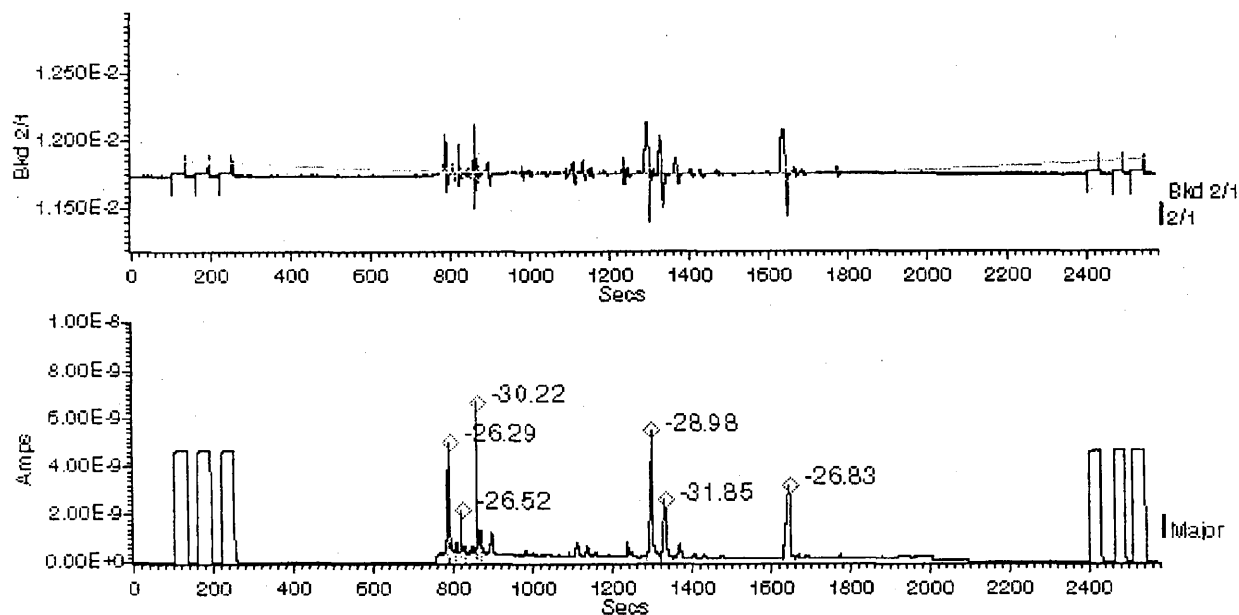
54

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_009 Folder : 230706
 Date : 23/07/06 Time : 12:24:59
 Comment : 176/07 995474 F3/45ul inj 2ul :
 Parameters Automatic DP Params

× **Data Processing Main Graph** □ □

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

55

auto

Data Processing Results

Data File Name : DATA_009
 Folder : 230706
 Sample Name : 178/07 995474 F3/45ul inj 2ul
 Sample ID :
 Sample Position : 4
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 12:24:59 Date : 23/07/06
 Current Time : 17:37:10 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.494E-8 | 1.1782E-2 | 4.2529E-3 |
| 182.6 | 8.494E-8 | 1.1782E-2 | 4.2530E-3 |
| 242.6 | 8.494E-8 | 1.1781E-2 | 4.2527E-3 |
| 2423.5 | 8.443E-8 | 1.1780E-2 | 4.2518E-3 |
| 2483.5 | 8.453E-8 | 1.1779E-2 | 4.2517E-3 |
| 2533.5 | 8.566E-8 | 1.1779E-2 | 4.2520E-3 |

Std Dev Of Fit 5.6232E-7 1.7469E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 795.1 | 5.07E-9 | 2.5669E-8 | 1.1854E-2 | 4.2100E-3 | -27.72 | -40.53 | -29.16 | 5 |
| | 827.1 | 2.21E-9 | 1.0385E-8 | 1.1849E-2 | 4.2270E-3 | -28.36 | -41.89 | -25.22 | 6 |
| | 867.4 | 6.75E-9 | 2.9980E-8 | 1.1814E-2 | 4.1853E-3 | -31.04 | -43.63 | -34.83 | 7 |
| | 1304.7 | 5.53E-9 | 4.9528E-8 | 1.1839E-2 | 4.1681E-3 | -28.67 | -53.91 | -38.76 | 11 |
| | 1337.2 | 2.59E-9 | 2.3118E-8 | 1.1802E-2 | 4.1708E-3 | -31.93 | -54.01 | -38.13 | 12 |
| | 1652.0 | 3.25E-9 | 3.7635E-8 | 1.1860E-2 | 4.1682E-3 | -26.83 | -50.14 | -38.72 | 12 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

56

GDC00925

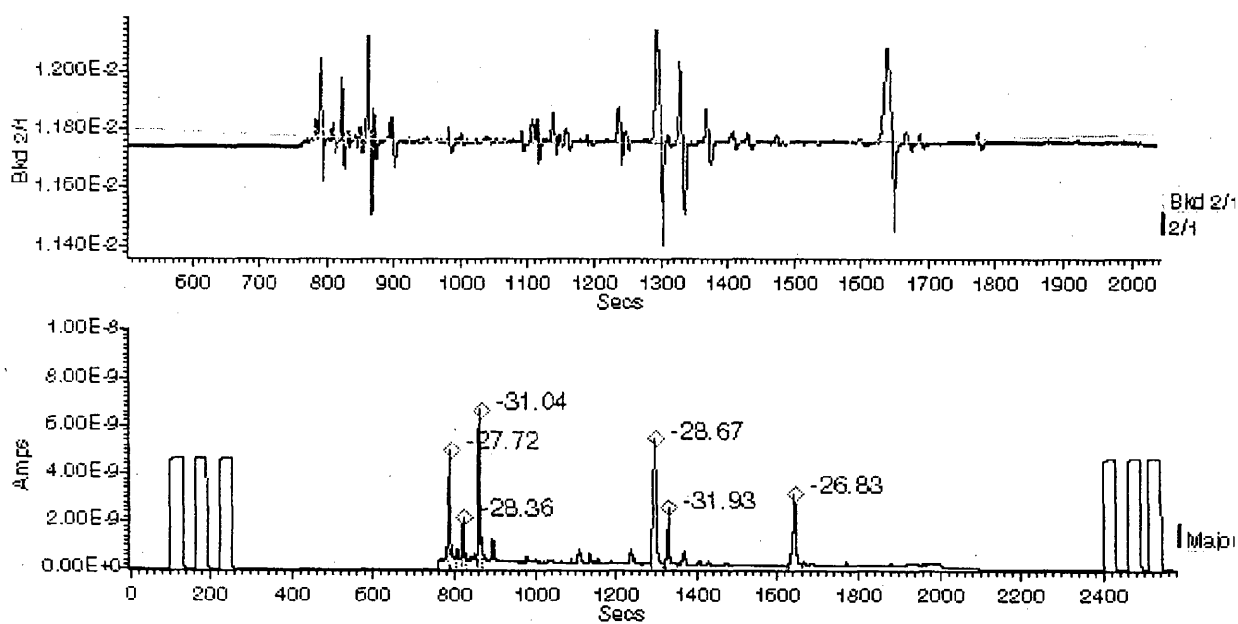
DP **Optima GC 1.67-2 - Manual DP**

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_009 Folder : 230706
 Date : 23/07/06 Time : 12:24:59
 Comment : 178/07 995474 F3/45ul inj 2ul :
 Parameters : Automatic DP Params

⌂ **Data Processing Main Graph**

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

auto

Data File Name : DATA_010
 Folder : 230706
 Sample Name : Blu 1 pool 4 F1/50ul inj 1ul
 Sample ID :
 Sample Position : 5
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 13:11:34 Date : 23/07/06
 Current Time : 17:45:24 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.633E-8 | 1.1780E-2 | 4.2529E-3 |
| 182.6 | 8.640E-8 | 1.1780E-2 | 4.2531E-3 |
| 242.7 | 8.674E-8 | 1.1780E-2 | 4.2530E-3 |
| 2423.5 | 8.603E-8 | 1.1779E-2 | 4.2523E-3 |
| 2483.5 | 8.553E-8 | 1.1779E-2 | 4.2524E-3 |
| 2533.5 | 8.673E-8 | 1.1780E-2 | 4.2528E-3 |

Std Dev Of Fit 1.5656E-7 1.9660E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 867.0 | 3.68E-9 | 1.5842E-8 | 1.1815E-2 | 4.1787E-3 | -30.80 | -58.95 | -36.42 | 15 |
| 1473.5 | 3.27E-9 | 3.4193E-8 | 1.1885E-2 | 4.1658E-3 | -24.55 | -59.30 | -39.40 | 18 |
| 1763.2 | 1.29E-9 | 1.4952E-8 | 1.1865E-2 | 4.1785E-3 | -26.44 | -58.27 | -36.43 | 17 |
| 1826.6 | 3.77E-9 | 4.6201E-8 | 1.1879E-2 | 4.1729E-3 | -25.16 | -57.89 | -37.73 | 16 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

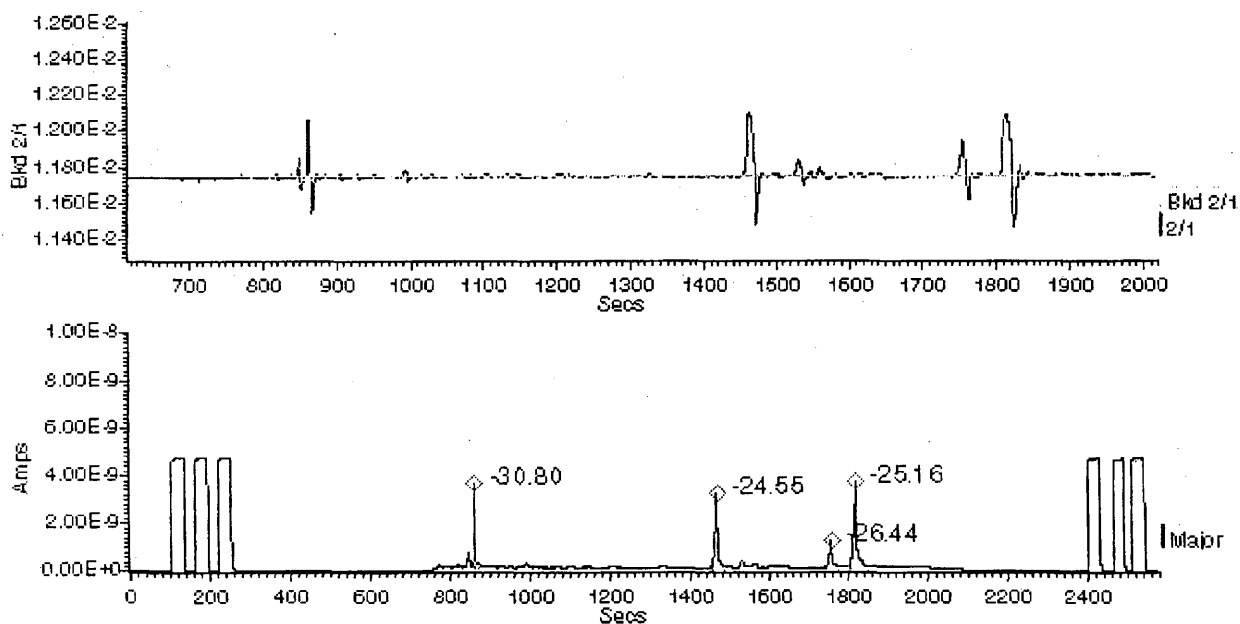
58

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_010 Folder : 230706
 Date : 23/07/06 Time : 13:11:34
 Comment : Blu 1 pool 4 F1/50ul inj 1ul :
 Parameters Automatic DP Params

≡ **Data Processing Main Graph** □ □

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

59

Data Processing Results

Manual.

Data File Name : DATA_010
 Folder : 230706
 Sample Name : Blu 1 pool 4 F1/50ul inj 1ul
 Sample ID :
 Sample Position : 5
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 13:11:34 Date : 23/07/06
 Current Time : 17:46:48 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.633E-8 | 1.1780E-2 | 4.2529E-3 |
| 182.6 | 8.640E-8 | 1.1780E-2 | 4.2531E-3 |
| 242.7 | 8.674E-8 | 1.1780E-2 | 4.2530E-3 |
| 2423.5 | 8.603E-8 | 1.1779E-2 | 4.2523E-3 |
| 2483.5 | 8.553E-8 | 1.1779E-2 | 4.2524E-3 |
| 2533.5 | 8.673E-8 | 1.1780E-2 | 4.2528E-3 |

Std Dev Of Fit 1.5656E-7 1.9660E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18Bkd |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|---------|
| 867.0 | 3.68E-9 | 1.5842E-8 | 1.1815E-2 | 4.1787E-3 | -30.80 | -58.95 | -36.42 | -59.40 |
| 1473.5 | 3.27E-9 | 3.4193E-8 | 1.1886E-2 | 4.1658E-3 | -24.49 | -59.65 | -39.40 | -59.40 |
| 1763.2 | 1.29E-9 | 1.4952E-8 | 1.1870E-2 | 4.1785E-3 | -25.97 | -59.40 | -36.43 | -59.40 |
| 1826.6 | 3.77E-9 | 4.6201E-8 | 1.1882E-2 | 4.1729E-3 | -24.92 | -59.24 | -37.73 | -59.40 |

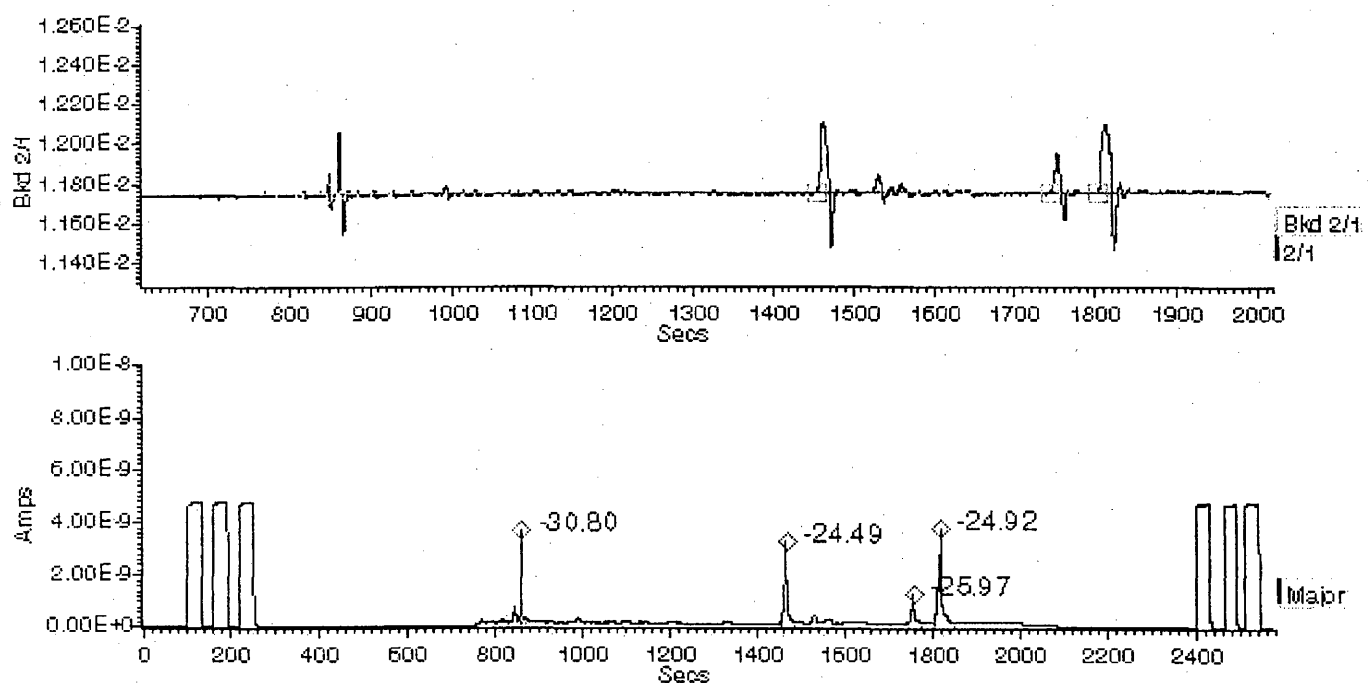
COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

60

Data Filename : DATA_010 Folder : 230706
 Date : 23/07/06 Time : 13:11:34
 Comment : Blu 1 pool 4 F1/50ul inj 1ul:
 Parameters Automatic DP Params

√ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

61

Data Processing Results

Zero

Data File Name : DATA_010
 Folder : 230706
 Sample Name : Blu 1 pool 4 F1/50ul inj 1ul
 Sample ID :
 Sample Position : 5
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 13:11:34 Date : 23/07/06
 Current Time : 17:47:42 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.633E-8 | 1.1780E-2 | 4.2529E-3 |
| 182.6 | 8.640E-8 | 1.1780E-2 | 4.2531E-3 |
| 242.7 | 8.674E-8 | 1.1780E-2 | 4.2530E-3 |
| 2423.5 | 8.603E-8 | 1.1779E-2 | 4.2523E-3 |
| 2483.5 | 8.553E-8 | 1.1779E-2 | 4.2524E-3 |
| 2533.5 | 8.673E-8 | 1.1780E-2 | 4.2528E-3 |

Std Dev Of Fit 1.5656E-7 1.9660E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 867.0 | 3.68E-9 | 1.5842E-8 | 1.1817E-2 | 4.1666E-3 | -30.54 | -39.22 > |
| 1473.5 | 3.27E-9 | 3.4193E-8 | 1.1880E-2 | 4.1670E-3 | -25.03 | -39.11<> |
| 1763.2 | 1.29E-9 | 1.4952E-8 | 1.1862E-2 | 4.1664E-3 | -26.61 | -39.23<> |
| 1826.6 | 3.77E-9 | 4.6201E-8 | 1.1876E-2 | 4.1651E-3 | -25.34 | -39.54< |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

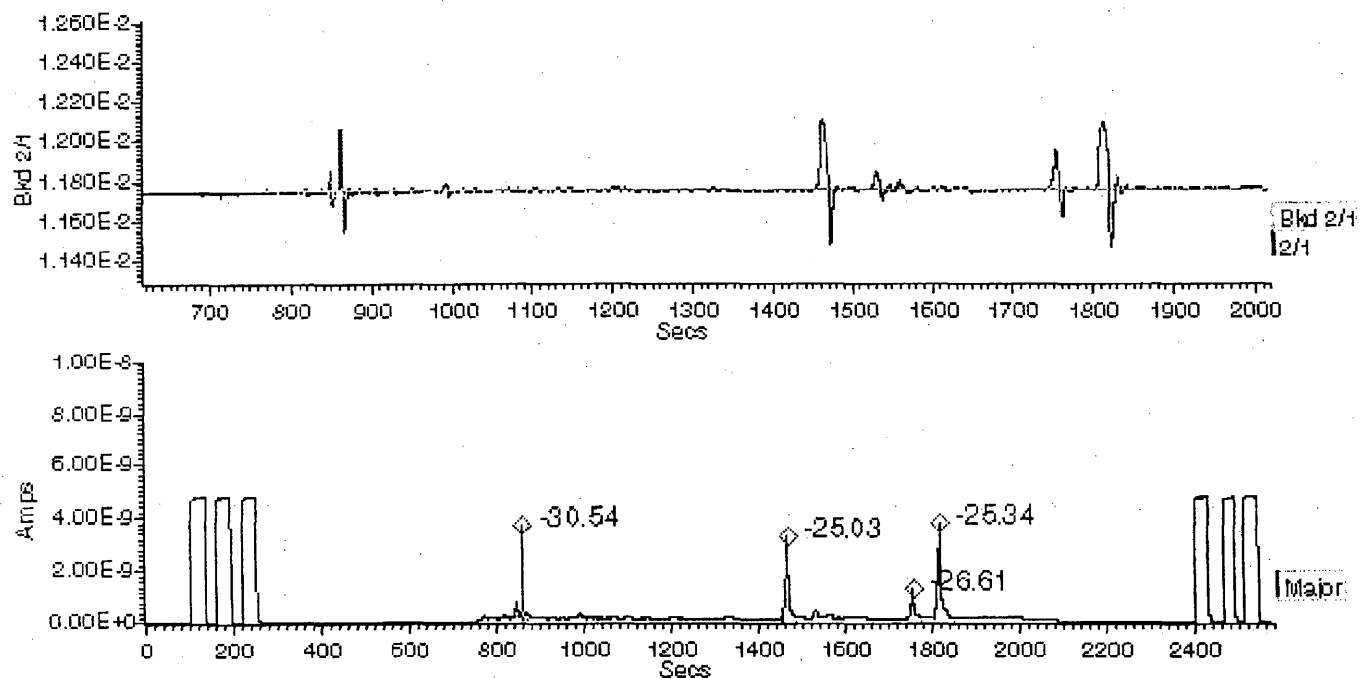
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename: DATA_010 Folder: 230706
 Date: 23/07/06 Time: 13:11:34
 Comment: Blu 1 pool 4 F1/50ul inj 1ul:
 Parameters Automatic DP Params

x Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

63

Data Processing Results

auto

Data File Name : DATA_011
 Folder : 230706
 Sample Name : 178/07 995474 F1/50ul inj 1ul
 Sample ID :
 Sample Position : 6
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 13:56:22 Date : 23/07/06
 Current Time : 17:49:11 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.557E-8 | 1.1780E-2 | 4.2530E-3 |
| 182.6 | 8.541E-8 | 1.1779E-2 | 4.2529E-3 |
| 242.7 | 8.559E-8 | 1.1779E-2 | 4.2528E-3 |
| 2423.5 | 8.570E-8 | 1.1780E-2 | 4.2528E-3 |
| 2483.5 | 8.477E-8 | 1.1779E-2 | 4.2526E-3 |
| 2533.5 | 8.552E-8 | 1.1779E-2 | 4.2525E-3 |

Std Dev Of Fit

4.6711E-7

1.1648E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 778.0 | 1.02E-8 | 5.1128E-8 | 1.1785E-2 | 4.1817E-3 | -33.44 | -54.70 | -35.72 | 5 |
| | 867.0 | 4.02E-9 | 2.3358E-8 | 1.1803E-2 | 4.1997E-3 | -32.02 | -52.64 | -31.57 | 6 |
| | 881.6 | 2.01E-9 | 1.9104E-8 | 1.1774E-2 | 4.2233E-3 | -34.70 | -52.23 | -26.11 | 6 |
| | 1236.3 | 1.47E-9 | 1.4752E-8 | 1.1838E-2 | 4.2016E-3 | -28.92 | -47.52 | -31.14 | 9 |
| | 1478.2 | 4.55E-9 | 5.1339E-8 | 1.1882E-2 | 4.1691E-3 | -24.87 | -47.26 | -38.62 | 10 |
| | 1764.6 | 1.56E-9 | 1.7786E-8 | 1.1874E-2 | 4.1715E-3 | -25.54 | -50.13 | -38.06 | 10 |
| | 1824.1 | 2.65E-9 | 2.5363E-8 | 1.1884E-2 | 4.1684E-3 | -24.66 | -51.10 | -38.77 | 10 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

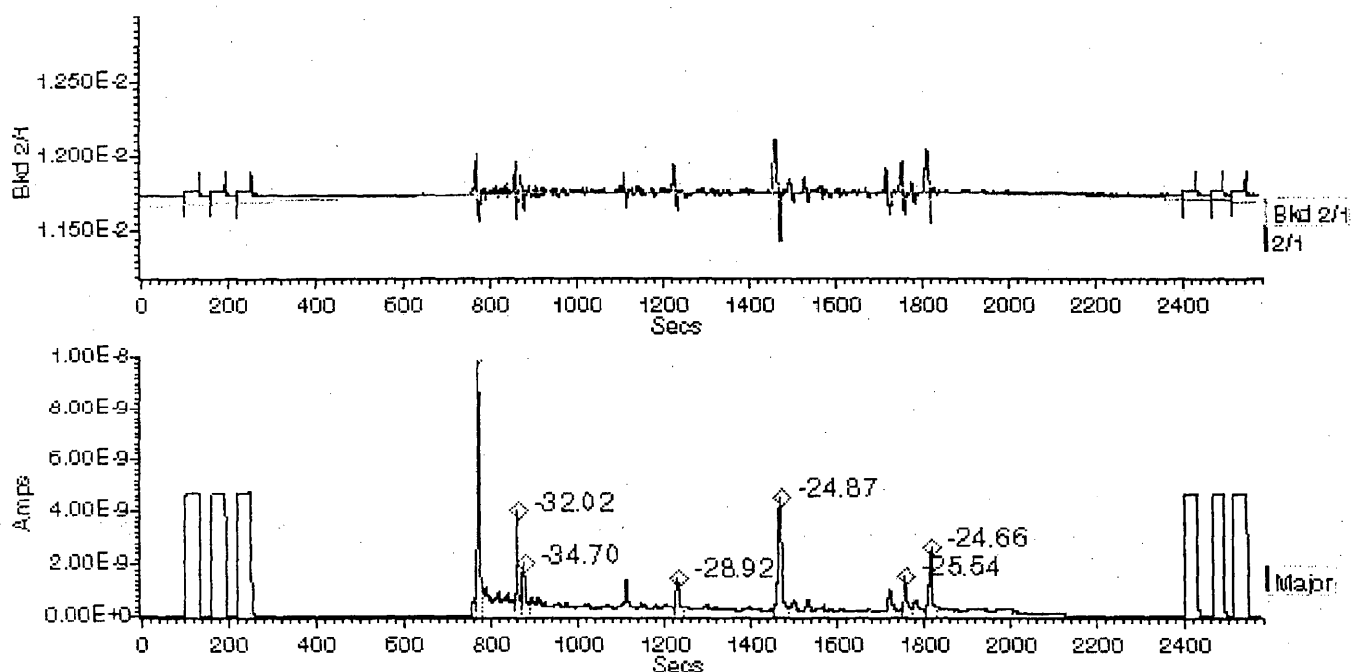
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_011 Folder : 230706
 Date : 23/07/06 Time : 13:56:22
 Comment : 178/07 995474 F1/50ul inj 1ul:
 Parameters Automatic DP Params

≤ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

65

Data Processing Results

Manual.

Data File Name : DATA_011
 Folder : 230706
 Sample Name : 178/07 995474 F1/50ul inj 1ul
 Sample ID :
 Sample Position : 6
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 13:56:22 Date : 23/07/06
 Current Time : 17:55:04 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.557E-8 | 1.1780E-2 | 4.2530E-3 |
| 182.6 | 8.541E-8 | 1.1779E-2 | 4.2529E-3 |
| 242.7 | 8.559E-8 | 1.1779E-2 | 4.2528E-3 |
| 2423.5 | 8.570E-8 | 1.1780E-2 | 4.2528E-3 |
| 2483.5 | 8.477E-8 | 1.1779E-2 | 4.2526E-3 |
| 2533.5 | 8.552E-8 | 1.1779E-2 | 4.2525E-3 |

Std Dev Of Fit 4.6711E-7 1.1648E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 778.0 | 1.02E-8 | 5.1128E-8 | 1.1780E-2 | 4.1817E-3 | -33.87 | -48.88 | -35.72 | 5 |
| | 867.0 | 4.02E-9 | 2.2259E-8 | 1.1797E-2 | 4.1959E-3 | -32.54 | -49.21 | -32.45 | 6 |
| | 881.6 | 2.01E-9 | 1.9104E-8 | 1.1765E-2 | 4.2233E-3 | -35.56 | -49.23 | -26.10 | 6 |
| | 1236.3 | 1.47E-9 | 1.4752E-8 | 1.1852E-2 | 4.2016E-3 | -27.71 | -50.15 | -31.14 | 9 |
| | 1478.2 | 4.55E-9 | 5.1339E-8 | 1.1888E-2 | 4.1691E-3 | -24.30 | -50.48 | -38.62 | 10 |
| | 1764.6 | 1.56E-9 | 1.7786E-8 | 1.1877E-2 | 4.1715E-3 | -25.28 | -50.64 | -38.06 | 10 |
| | 1824.1 | 2.65E-9 | 2.5363E-8 | 1.1883E-2 | 4.1684E-3 | -24.78 | -50.63 | -38.77 | 10 |

COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

DP Optima GC 1.67-2 - Manual DP

□ □

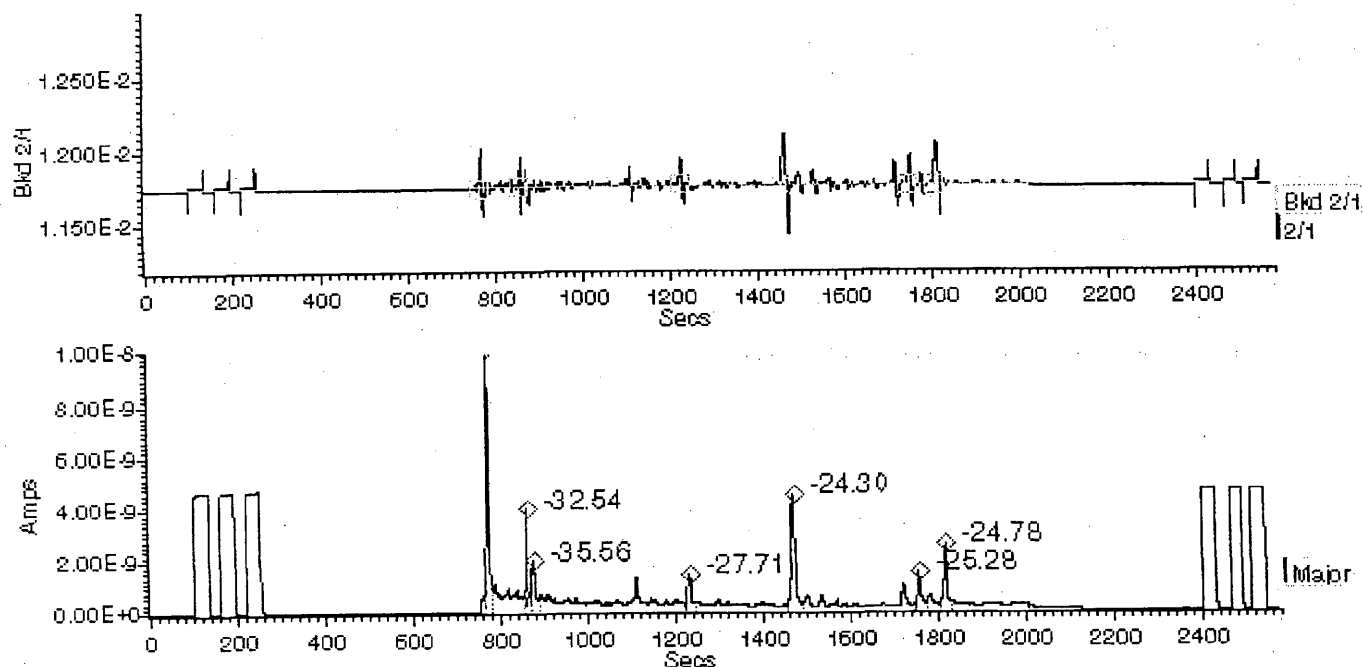
File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_011 Folder : 230706
 Date : 23/07/06 Time : 13:56:22
 Comment : 178/07 995474 F1/50ul inj 1ul:
 Parameters Automatic DP Params

◀ Data Processing Main Graph

□ □

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINALS

67

Data Processing Results

Zero

Data File Name : DATA_011
 Folder : 230706
 Sample Name : 178/07 995474 F1/50ul inj 1ul
 Sample ID :
 Sample Position : 6
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 13:56:22 Date : 23/07/06
 Current Time : 17:55:51 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.557E-8 | 1.1780E-2 | 4.2530E-3 |
| 182.6 | 8.541E-8 | 1.1779E-2 | 4.2529E-3 |
| 242.7 | 8.559E-8 | 1.1779E-2 | 4.2528E-3 |
| 2423.5 | 8.570E-8 | 1.1780E-2 | 4.2528E-3 |
| 2483.5 | 8.477E-8 | 1.1779E-2 | 4.2526E-3 |
| 2533.5 | 8.552E-8 | 1.1779E-2 | 4.2525E-3 |

Std Dev Of Fit 4.6711E-7 1.1648E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 778.0 | 1.02E-8 | 5.1128E-8 | 1.1783E-2 | 4.1647E-3 | -33.51 | -39.64 > |
| 867.0 | 4.02E-9 | 2.3358E-8 | 1.1800E-2 | 4.1667E-3 | -32.03 | -39.18<> |
| 881.6 | 2.01E-9 | 1.9104E-8 | 1.1776E-2 | 4.1689E-3 | -34.11 | -38.66<> |
| 1236.3 | 1.47E-9 | 1.4752E-8 | 1.1845E-2 | 4.1690E-3 | -28.09 | -38.66<> |
| 1478.2 | 4.55E-9 | 5.1339E-8 | 1.1880E-2 | 4.1653E-3 | -24.96 | -39.51<> |
| 1764.6 | 1.56E-9 | 1.7786E-8 | 1.1862E-2 | 4.1686E-3 | -26.58 | -38.73<> |
| 1824.1 | 2.65E-9 | 2.5363E-8 | 1.1873E-2 | 4.1670E-3 | -25.65 | -39.10< |

COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

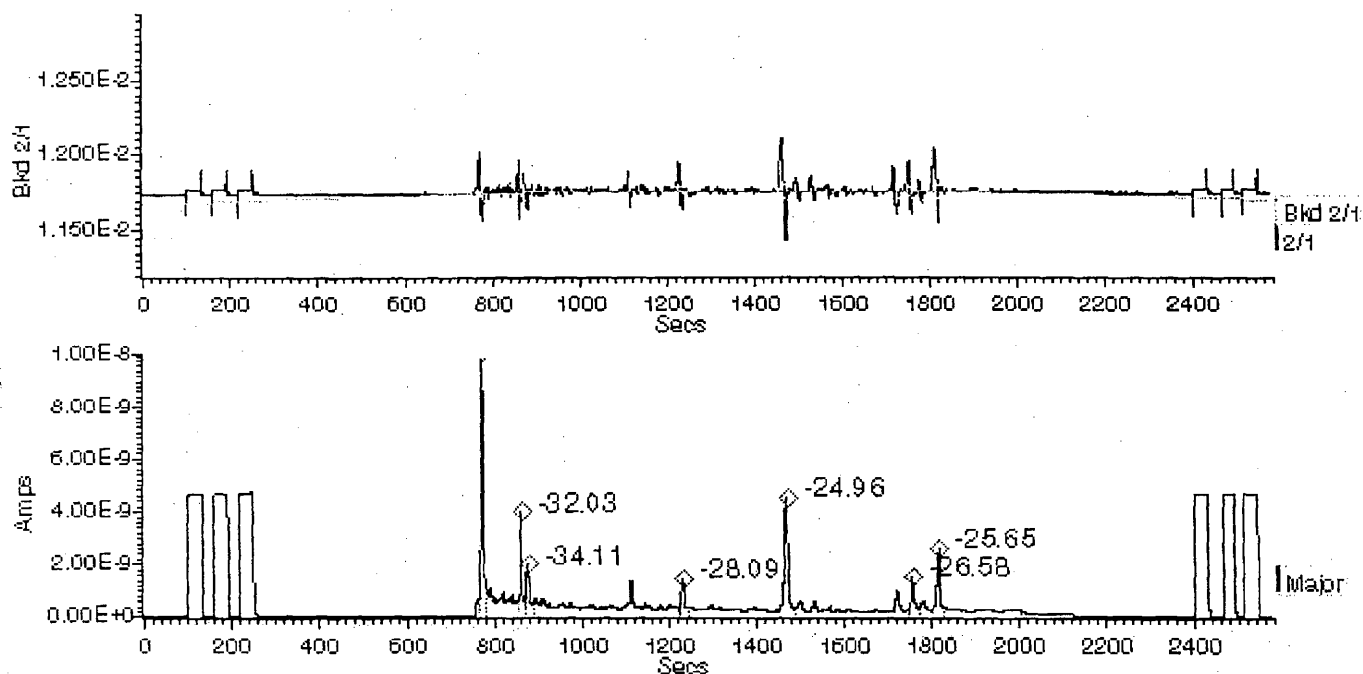
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_011 Folder : 230706
 Date : 23/07/06 Time : 13:56:22
 Comment : 178/07 995474 F1/50ul inj 1ul :
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

69

Data Processing Results

Auto

Data File Name : DATA_012
 Folder : 230706
 Sample Name : Blu 1 pool 4 F2/500ul inj 1ul
 Sample ID :
 Sample Position : 7
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 14:41:06 Date : 23/07/06
 Current Time : 17:57:46 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.634E-8 | 1.1779E-2 | 4.2525E-3 |
| 182.6 | 8.646E-8 | 1.1779E-2 | 4.2529E-3 |
| 242.6 | 8.648E-8 | 1.1779E-2 | 4.2529E-3 |
| 2423.4 | 8.572E-8 | 1.1779E-2 | 4.2526E-3 |
| 2483.4 | 8.565E-8 | 1.1778E-2 | 4.2526E-3 |
| 2533.5 | 8.561E-8 | 1.1779E-2 | 4.2529E-3 |

Std Dev Of Fit 2.0409E-7 1.7605E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 868.0 | 2.74E-9 | 1.4913E-8 | 1.1823E-2 | 4.1667E-3 | -29.98 | -65.41 | -39.17 | 21 |
| 1231.7 | 4.52E-9 | 3.6233E-8 | 1.1880E-2 | 4.1674E-3 | -25.01 | -63.47 | -39.01 | 21 |
| 1257.2 | 5.33E-9 | 4.2320E-8 | 1.1880E-2 | 4.1673E-3 | -24.99 | -63.27 | -39.04 | 20 |

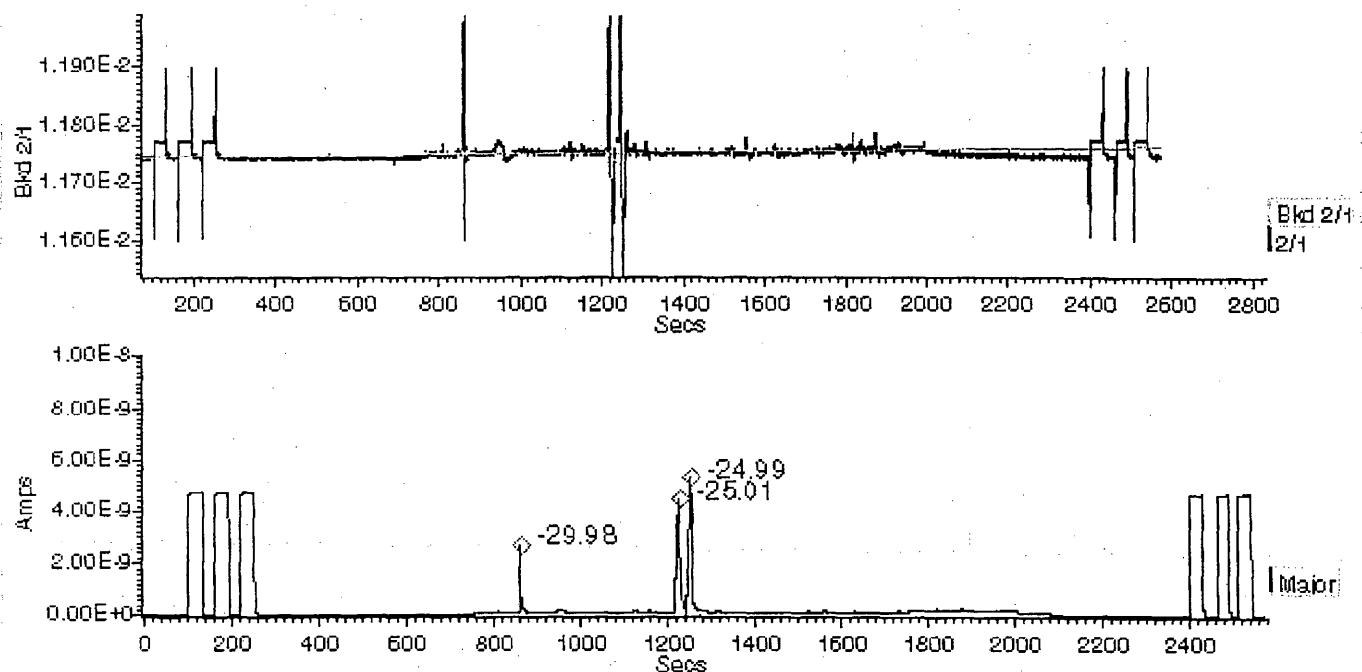
COPIE CERTIFIÉE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_012 Folder : 230706
 Date : 23/07/06 Time : 14:41:06
 Comment : Blu 1.pool 4 F2/500ul inj 1ul :
 Parameters Automatic DP Params

< Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

71

Data Processing Results

manual.

Data File Name : DATA_012
 Folder : 230706
 Sample Name : Blu 1 pool 4 F2/500ul inj 1ul
 Sample ID :
 Sample Position : 7
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 14:41:06 Date : 23/07/06
 Current Time : 17:59:36 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.634E-8 | 1.1779E-2 | 4.2525E-3 |
| 182.6 | 8.646E-8 | 1.1779E-2 | 4.2529E-3 |
| 242.6 | 8.648E-8 | 1.1779E-2 | 4.2529E-3 |
| 2423.4 | 8.572E-8 | 1.1779E-2 | 4.2526E-3 |
| 2483.4 | 8.565E-8 | 1.1778E-2 | 4.2526E-3 |
| 2533.5 | 8.561E-8 | 1.1779E-2 | 4.2529E-3 |

Std Dev Of Fit 2.0409E-7 1.7605E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 868.0 | 2.74E-9 | 1.2184E-8 | 1.1824E-2 | 4.1671E-3 | -29.86 | -65.60 | -39.08 | 21 |
| | 1231.7 | 4.52E-9 | 3.6233E-8 | 1.1880E-2 | 4.1674E-3 | -25.00 | -63.56 | -39.01 | 21 |
| | 1257.2 | 5.33E-9 | 4.2320E-8 | 1.1880E-2 | 4.1673E-3 | -24.97 | -63.43 | -39.04 | 20 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

DP **Optima GC 1.67-2 - Manual DP**

□ □

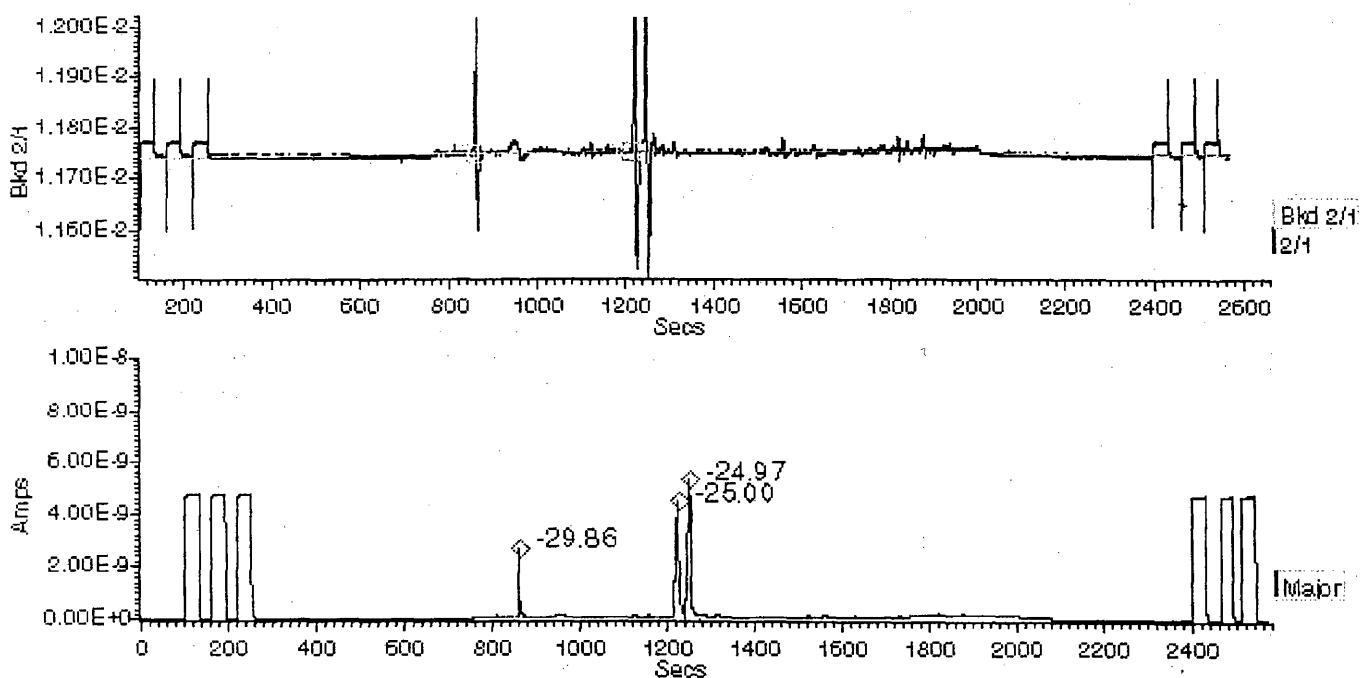
File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_012 Folder : 230706
 Date : 23/07/06 Time : 14:41:06
 Comment : Blu 1 pool 4 F2/500ul inj 1ul:
 Parameters Automatic DP Params

⌵ **Data Processing Main Graph**

□ □

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

73

Data Processing Results

Zero

Data File Name : DATA_012
 Folder : 230706
 Sample Name : Blu 1 pool 4 F2/500ul inj 1ul
 Sample ID :
 Sample Position : 7
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 14:41:06 Date : 23/07/06
 Current Time : 18:00:22 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.634E-8 | 1.1779E-2 | 4.2525E-3 |
| 182.6 | 8.646E-8 | 1.1779E-2 | 4.2529E-3 |
| 242.6 | 8.648E-8 | 1.1779E-2 | 4.2529E-3 |
| 2423.4 | 8.572E-8 | 1.1779E-2 | 4.2526E-3 |
| 2483.4 | 8.565E-8 | 1.1778E-2 | 4.2526E-3 |
| 2533.5 | 8.561E-8 | 1.1779E-2 | 4.2529E-3 |

Std Dev Of Fit 2.0409E-7 1.7605E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 868.0 | 2.74E-9 | 1.4913E-8 | 1.1825E-2 | 4.1695E-3 | -29.81 | -38.53 > |
| 1231.7 | 4.52E-9 | 3.6233E-8 | 1.1879E-2 | 4.1655E-3 | -25.08 | -39.46 > |
| 1257.2 | 5.33E-9 | 4.2320E-8 | 1.1879E-2 | 4.1650E-3 | -25.05 | -39.57 < |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

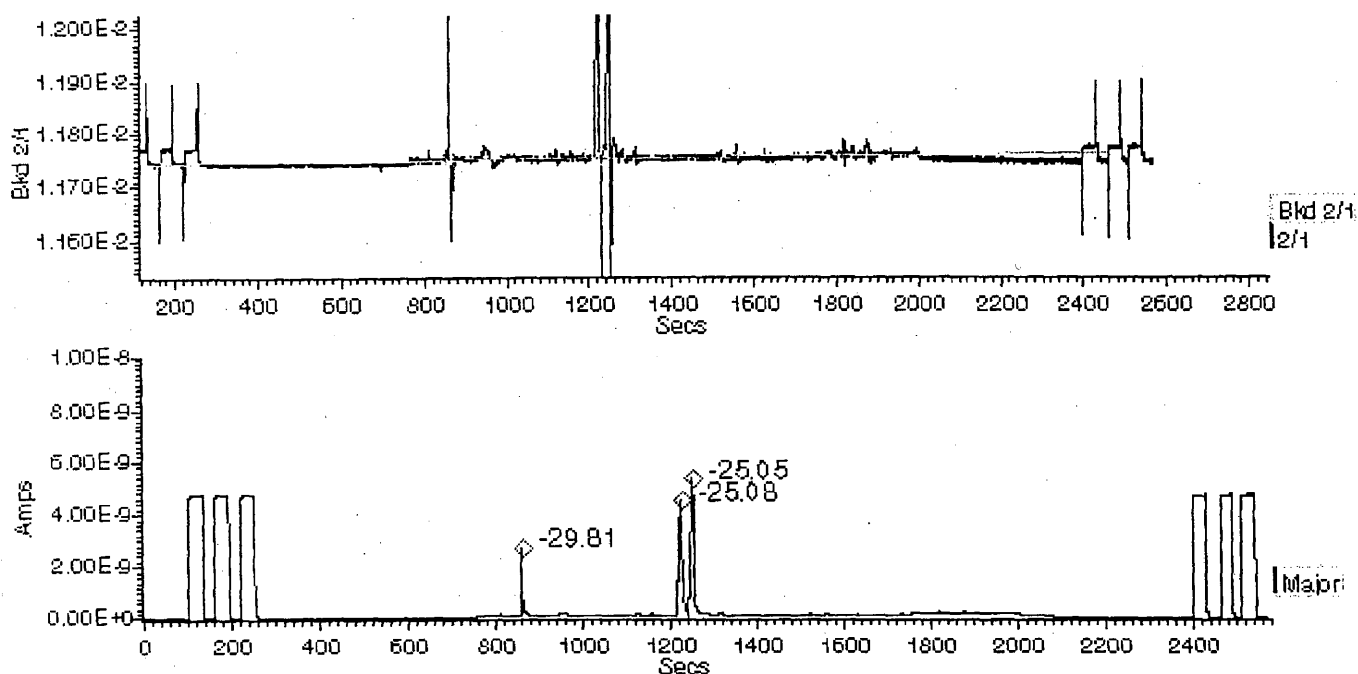
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename: DATA_012 Folder: 230706
 Date: 23/07/06 Time: 14:41:06
 Comment: Blu 1 pool 4 F2/500ul inj 1ul:
 Parameters Automatic DP Params

√ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

75

Data Processing Results

auto

Data File Name : DATA_013
 Folder : 230706
 Sample Name : 178/07 995474 F2/400ul inj 1ul
 Sample ID :
 Sample Position : 8
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 15:25:49 Date : 23/07/06
 Current Time : 18:01:45 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.603E-8 | 1.1780E-2 | 4.2535E-3 |
| 182.6 | 8.588E-8 | 1.1779E-2 | 4.2537E-3 |
| 242.6 | 8.578E-8 | 1.1779E-2 | 4.2536E-3 |
| 2423.5 | 8.471E-8 | 1.1779E-2 | 4.2525E-3 |
| 2483.5 | 8.452E-8 | 1.1778E-2 | 4.2521E-3 |
| 2533.5 | 8.511E-8 | 1.1777E-2 | 4.2522E-3 |

Std Dev Of Fit

4.5694E-7

1.4227E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 866.0 | 2.17E-9 | 9.6593E-9 | 1.1822E-2 | 4.1703E-3 | -30.07 | -63.69 | -38.44 | 18 |
| 1229.7 | 3.95E-9 | 3.1256E-8 | 1.1863E-2 | 4.1661E-3 | -26.43 | -60.89 | -39.38 | 18 |
| 1254.3 | 3.39E-9 | 2.5932E-8 | 1.1849E-2 | 4.1704E-3 | -27.71 | -60.19 | -38.39 | 18 |
| 1288.3 | 3.55E-9 | 2.9815E-8 | 1.1797E-2 | 4.1768E-3 | -32.28 | -59.25 | -36.88 | 17 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

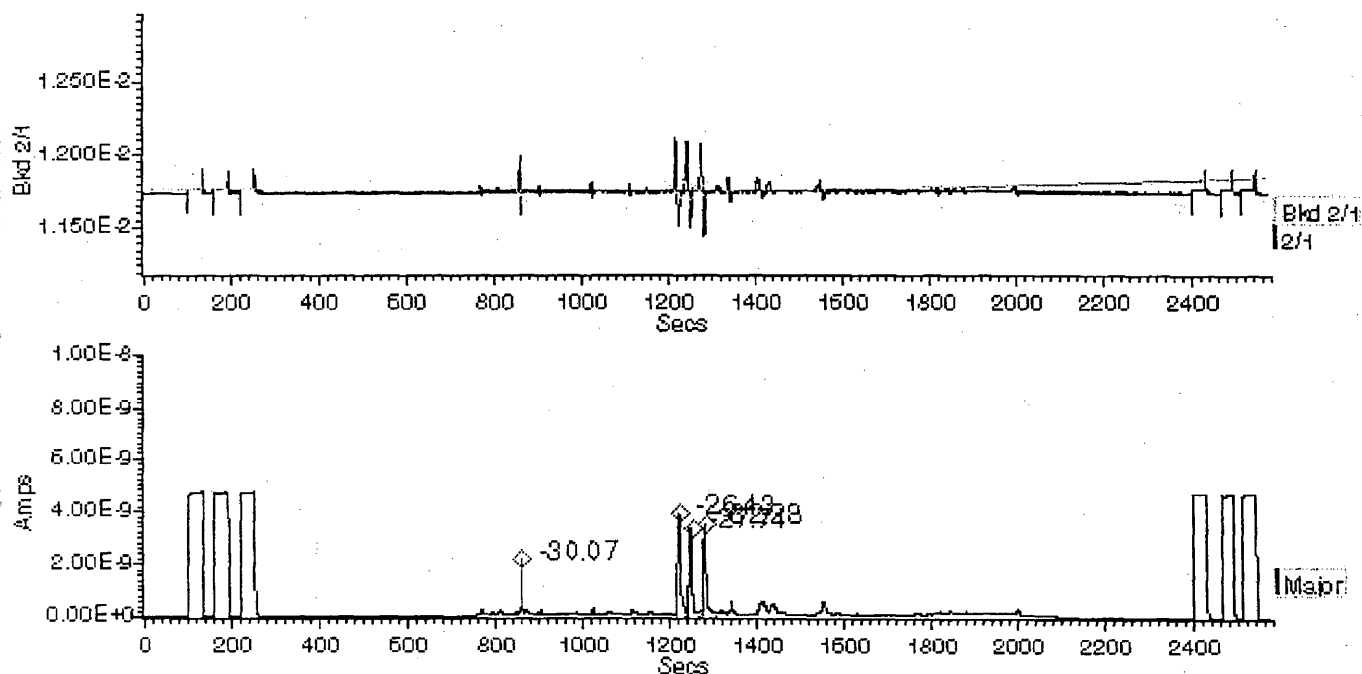
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_013 Folder : 230706
 Date : 23/07/06 Time : 15:25:49
 Comment : 178/07 995474 F2/400ul inj 1ul
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

77

Data Processing Results

Data File Name : DATA_013
 Folder : 230706
 Sample Name : 178/07 995474 F2/400ul inj 1ul
 Sample ID :
 Sample Position : 8
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 15:25:49 Date : 23/07/06
 Current Time : 18:29:49 Date : 04/05/07

Manual.

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.603E-8 | 1.1780E-2 | 4.2535E-3 |
| 182.6 | 8.588E-8 | 1.1779E-2 | 4.2537E-3 |
| 242.6 | 8.578E-8 | 1.1779E-2 | 4.2536E-3 |
| 2423.5 | 8.471E-8 | 1.1779E-2 | 4.2525E-3 |
| 2483.5 | 8.452E-8 | 1.1778E-2 | 4.2521E-3 |
| 2533.5 | 8.511E-8 | 1.1777E-2 | 4.2522E-3 |

Std Dev Of Fit 4.5694E-7 1.4227E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 866.0 | 2.17E-9 | 9.6593E-9 | 1.1822E-2 | 4.1703E-3 | -30.09 | -63.62 | -38.44 | 18 |
| | 1229.7 | 3.95E-9 | 3.1256E-8 | 1.1863E-2 | 4.1661E-3 | -26.40 | -61.05 | -39.38 | 18 |
| | 1254.3 | 3.39E-9 | 2.5545E-8 | 1.1850E-2 | 4.1699E-3 | -27.60 | -60.75 | -38.49 | 18 |
| | 1288.3 | 3.55E-9 | 2.9815E-8 | 1.1800E-2 | 4.1768E-3 | -32.08 | -60.31 | -36.88 | 17 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

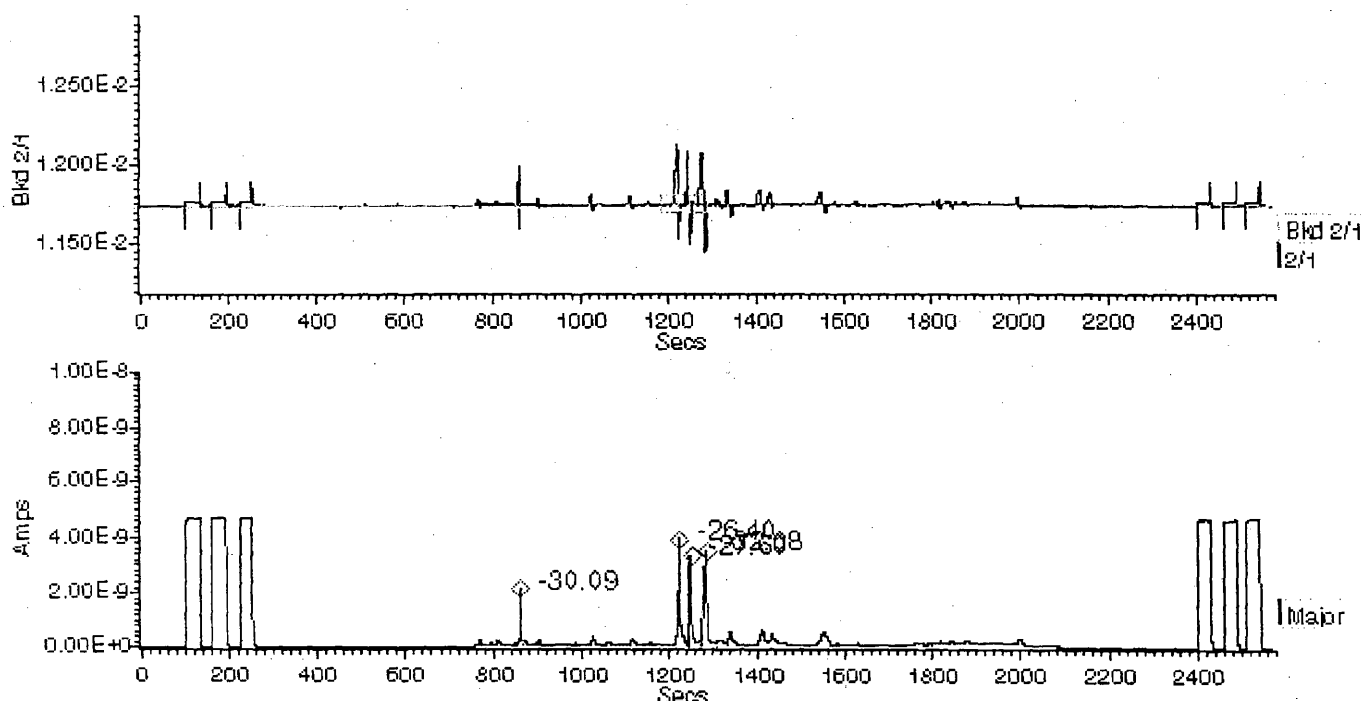
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_013 Folder : 230706
 Date : 23/07/06 Time : 15:25:49
 Comment : 178/07 995474 F2/400ul inj 1ul
 Parameters Automatic DP Params

≡ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

79

Data Processing Results

Zero

Data File Name : DATA_013
 Folder : 230706
 Sample Name : 178/07 995474 F2/400ul inj 1ul
 Sample ID :
 Sample Position : 8
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 15:25:49 Date : 23/07/06
 Current Time : 18:06:25 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.603E-8 | 1.1780E-2 | 4.2535E-3 |
| 182.6 | 8.588E-8 | 1.1779E-2 | 4.2537E-3 |
| 242.6 | 8.578E-8 | 1.1779E-2 | 4.2536E-3 |
| 2423.5 | 8.471E-8 | 1.1779E-2 | 4.2525E-3 |
| 2483.5 | 8.452E-8 | 1.1778E-2 | 4.2521E-3 |
| 2533.5 | 8.511E-8 | 1.1777E-2 | 4.2522E-3 |

Std Dev Of Fit 4.5694E-7 1.4227E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 866.0 | 2.17E-9 | 9.6593E-9 | 1.1820E-2 | 4.1683E-3 | -30.28 | -38.91 > |
| 1229.7 | 3.95E-9 | 3.1256E-8 | 1.1862E-2 | 4.1663E-3 | -26.55 | -39.34<> |
| 1254.3 | 3.39E-9 | 2.5932E-8 | 1.1850E-2 | 4.1666E-3 | -27.62 | -39.24<> |
| 1288.3 | 3.55E-9 | 2.9815E-8 | 1.1805E-2 | 4.1665E-3 | -31.50 | -39.26< |

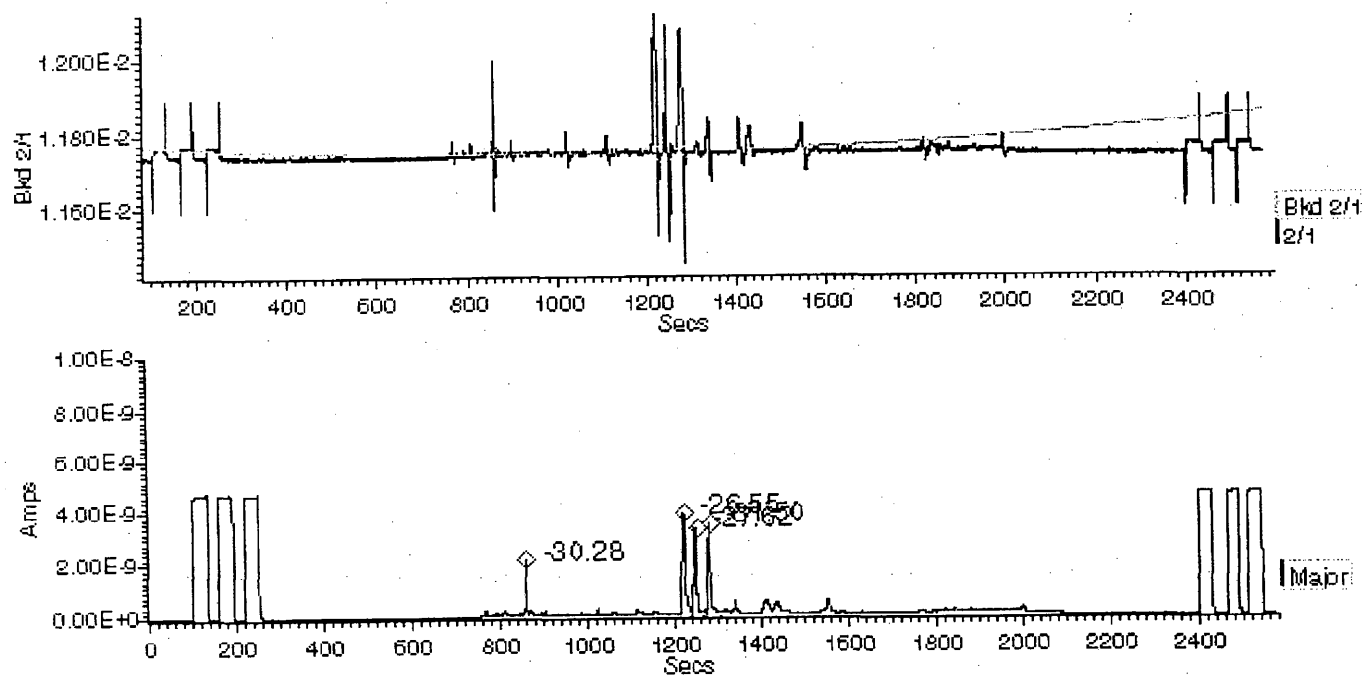
COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_013 Folder : 230706
 Date : 23/07/06 Time : 15:25:49
 Comment : 178/07 995474 F2/400ul inj 1ul
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

81

Data Processing Results

Data File Name : DATA_014
 Folder : 230706
 Sample Name : Mix Cal Acetate 001A-2
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name : 230706
 RunTime User : micromass
 Acquisition Time : 20:39:04 Date : 23/07/06
 Current Time : 18:07:36 Date : 04/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.663E-8 | 1.1777E-2 | 4.2533E-3 |
| 182.6 | 8.686E-8 | 1.1778E-2 | 4.2533E-3 |
| 242.6 | 8.696E-8 | 1.1777E-2 | 4.2538E-3 |
| 2423.5 | 8.634E-8 | 1.1776E-2 | 4.2523E-3 |
| 2483.5 | 8.613E-8 | 1.1776E-2 | 4.2525E-3 |
| 2533.4 | 8.650E-8 | 1.1776E-2 | 4.2530E-3 |

Std Dev Of Fit 2.3526E-7 3.3647E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 866.6 | 4.03E-9 | 1.7744E-8 | 1.1814E-2 | 4.1663E-3 | -30.58 | -69.02 | -39.38 | 24 |
| | 1228.5 | 4.07E-9 | 3.2515E-8 | 1.1931E-2 | 4.1699E-3 | -20.35 | -66.55 | -38.53 | 24 |
| | 1301.0 | 3.09E-9 | 2.5613E-8 | 1.1776E-2 | 4.1733E-3 | -33.95 | -66.44 | -37.70 | 24 |
| | 1471.6 | 2.60E-9 | 2.7183E-8 | 1.1970E-2 | 4.1718E-3 | -16.88 | -66.70 | -38.09 | 24 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

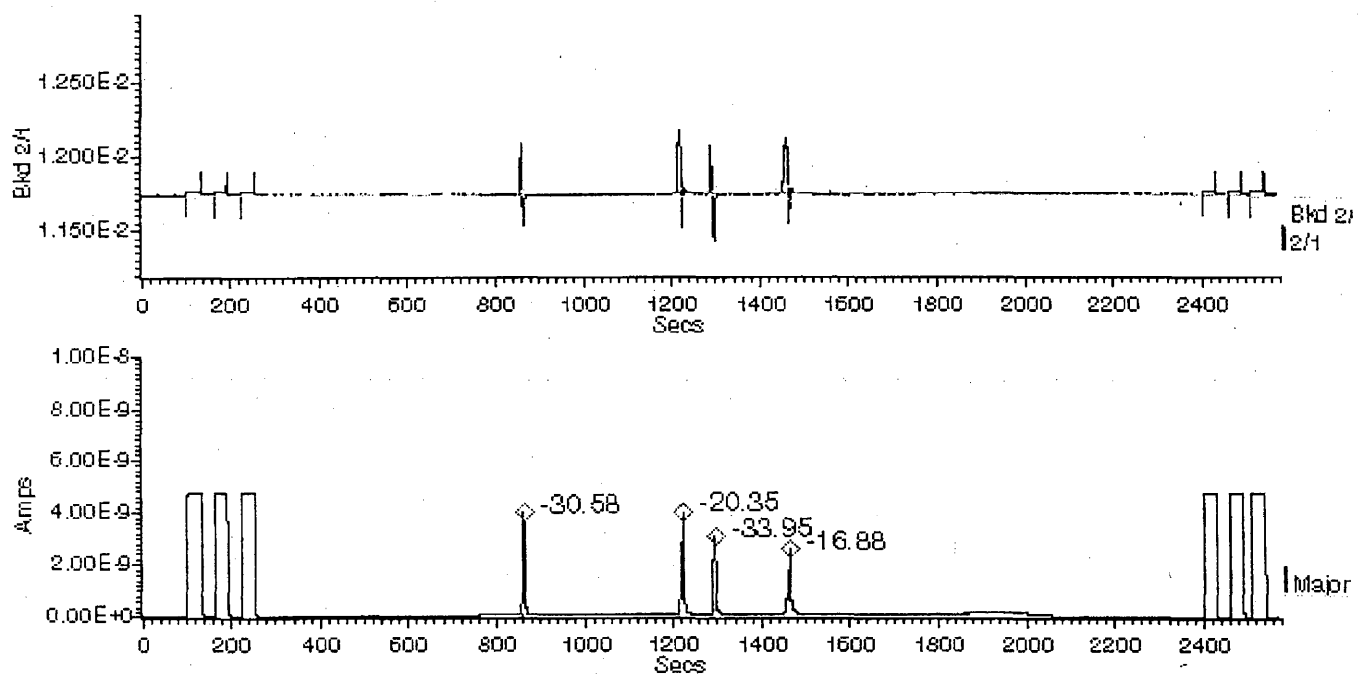
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_014 Folder : 230706
 Date : 23/07/06 Time : 20:39:04
 Comment : Mix Cal Acetate 001A-2 : 230706
 Parameters Automatic DP Params

× Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

GDC00952

83

Data Processing Results

Manual.

Data File Name : DATA_014
 Folder : 230706
 Sample Name : Mix Cal Acetate 001A-2
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name : 230706
 RunTime User : micromass
 Acquisition Time : 20:39:04 Date : 23/07/06
 Current Time : 18:11:00 Date : 04/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.663E-8 | 1.1777E-2 | 4.2533E-3 |
| 182.6 | 8.686E-8 | 1.1778E-2 | 4.2533E-3 |
| 242.6 | 8.696E-8 | 1.1777E-2 | 4.2538E-3 |
| 2423.5 | 8.634E-8 | 1.1776E-2 | 4.2523E-3 |
| 2483.5 | 8.613E-8 | 1.1776E-2 | 4.2525E-3 |
| 2533.4 | 8.650E-8 | 1.1776E-2 | 4.2530E-3 |

Std Dev Of Fit 2.3526E-7 3.3647E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 866.6 | 4.03E-9 | 1.7744E-8 | 1.1814E-2 | 4.1663E-3 | -30.58 | -69.02 | -39.38 | 24 |
| 1228.5 | 4.07E-9 | 3.1390E-8 | 1.1933E-2 | 4.1692E-3 | -20.12 | -67.31 | -38.69 | 24 |
| 1301.0 | 3.09E-9 | 2.3466E-8 | 1.1779E-2 | 4.1711E-3 | -33.63 | -67.13 | -38.22 | 24 |
| 1471.6 | 2.60E-9 | 2.7183E-8 | 1.1971E-2 | 4.1718E-3 | -16.86 | -66.81 | -38.09 | 24 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

84

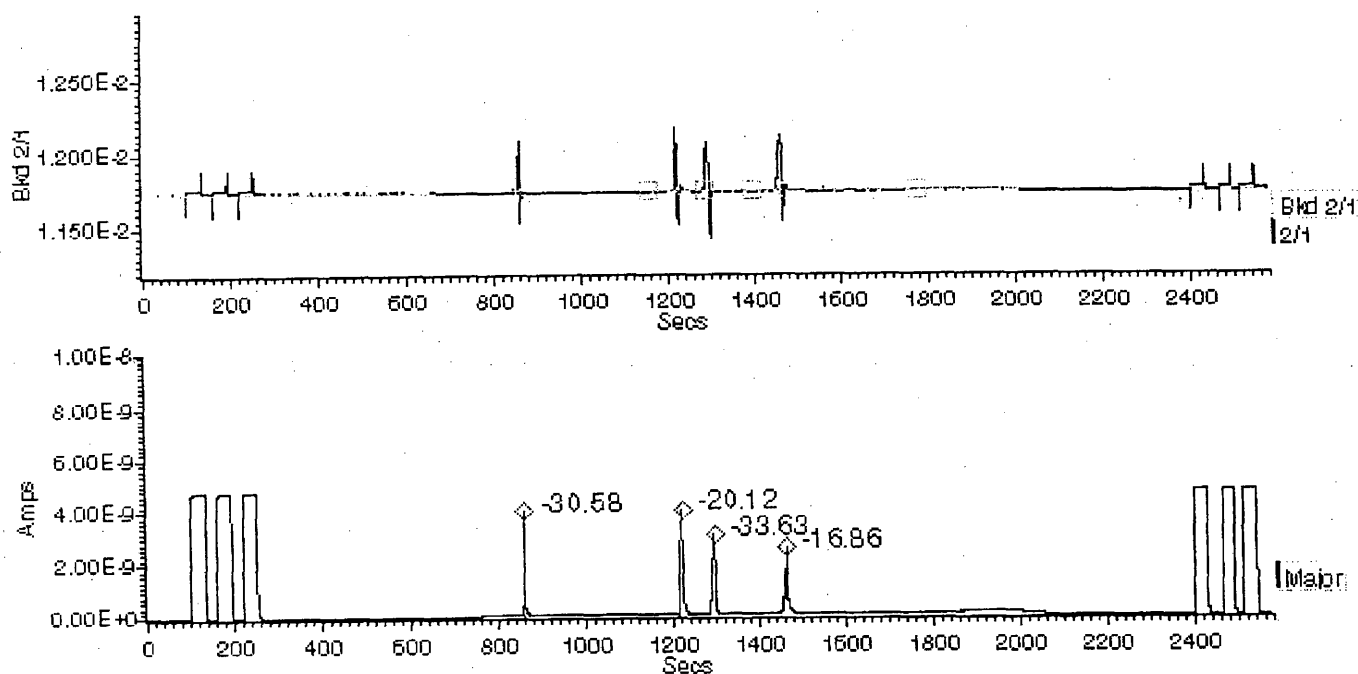
GDC00953

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_014 Folder : 230706
 Date : 23/07/06 Time : 20:39:04
 Comment : Mix Cal Acetate 001A-2 : 230706
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

85

Data Processing Results

Data File Name : DATA_014
 Folder : 230706
 Sample Name : Mix Cal Acetate 001A-2
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name : 230706
 RunTime User : micromass
 Acquisition Time : 20:39:04 Date : 23/07/06
 Current Time : 18:11:44 Date : 04/05/07

Zero

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.663E-8 | 1.1777E-2 | 4.2533E-3 |
| 182.6 | 8.686E-8 | 1.1778E-2 | 4.2533E-3 |
| 242.6 | 8.696E-8 | 1.1777E-2 | 4.2538E-3 |
| 2423.5 | 8.634E-8 | 1.1776E-2 | 4.2523E-3 |
| 2483.5 | 8.613E-8 | 1.1776E-2 | 4.2525E-3 |
| 2533.4 | 8.650E-8 | 1.1776E-2 | 4.2530E-3 |

Std Dev Of Fit 2.3526E-7 3.3647E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

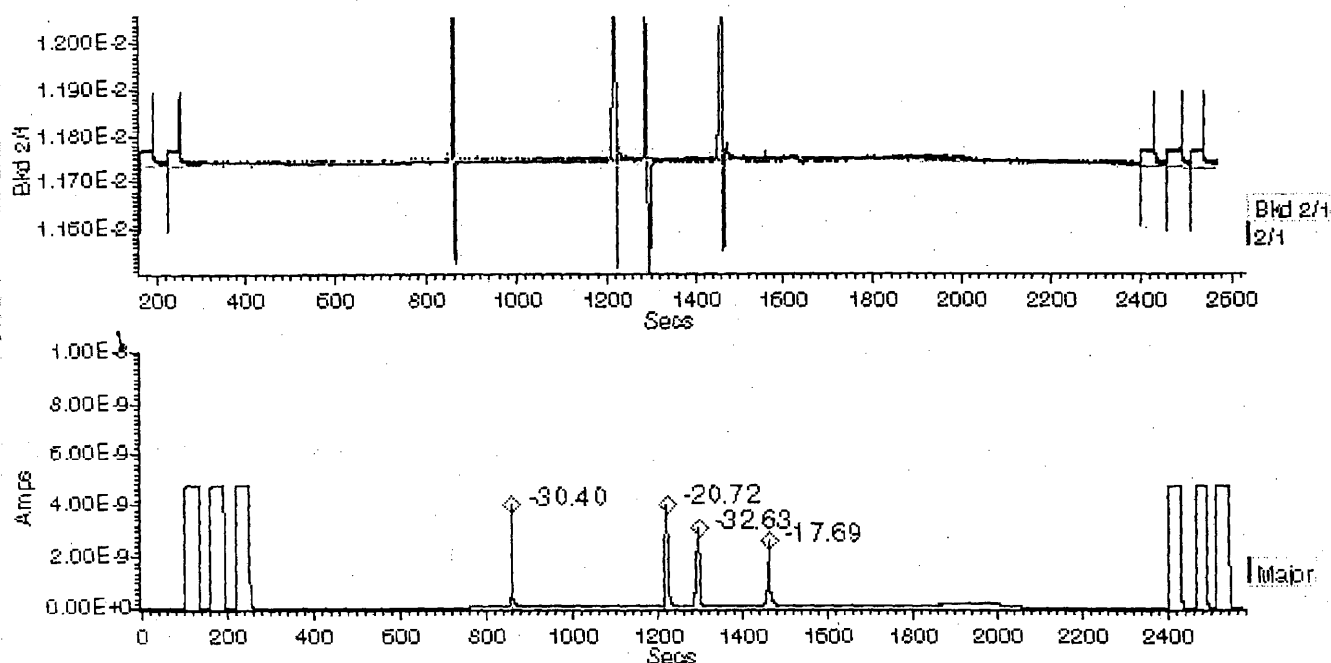
| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 866.6 | 4.03E-9 | 1.7744E-8 | 1.1816E-2 | 4.1652E-3 | -30.40 | -39.62 > |
| 1228.5 | 4.07E-9 | 3.2515E-8 | 1.1926E-2 | 4.1666E-3 | -20.72 | -39.30<> |
| 1301.0 | 3.09E-9 | 2.5613E-8 | 1.1790E-2 | 4.1677E-3 | -32.63 | -39.00<> |
| 1471.6 | 2.60E-9 | 2.7183E-8 | 1.1961E-2 | 4.1670E-3 | -17.69 | -39.17< |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Filename : DATA_014 Folder : 230706
 Date : 23/07/06 Time : 20:39:04
 Comment : Mix Cal Acetate 001A-2 : 230706
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

87

Data Processing Results

Manual

Data File Name : DATA_009
 Folder : 040806
 Sample Name : Mix Cal Acetate 001A-100ng inj
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 12:24:14 Date : 04/08/06
 Current Time : 09:55:28 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.507E-8 | 1.1776E-2 | 4.2528E-3 |
| 182.6 | 8.550E-8 | 1.1776E-2 | 4.2528E-3 |
| 242.6 | 8.493E-8 | 1.1776E-2 | 4.2530E-3 |
| 2423.4 | 8.422E-8 | 1.1775E-2 | 4.2523E-3 |
| 2483.5 | 8.354E-8 | 1.1775E-2 | 4.2518E-3 |
| 2533.5 | 8.443E-8 | 1.1775E-2 | 4.2521E-3 |

Std Dev Of Fit 9.2732E-8 1.7392E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 870.6 | 4.30E-9 | 1.9626E-8 | 1.1813E-2 | 4.1630E-3 | -30.55 | -75.50 | -39.98 | 32 |
| 1241.8 | 4.36E-9 | 3.6189E-8 | 1.1934E-2 | 4.1657E-3 | -19.99 | -74.56 | -39.35 | 31 |
| 1316.7 | 3.35E-9 | 2.8027E-8 | 1.1777E-2 | 4.1669E-3 | -33.73 | -74.36 | -39.05 | 31 |
| 1491.1 | 2.83E-9 | 3.2468E-8 | 1.1970E-2 | 4.1695E-3 | -16.86 | -73.80 | -38.46 | 31 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

GDC00957

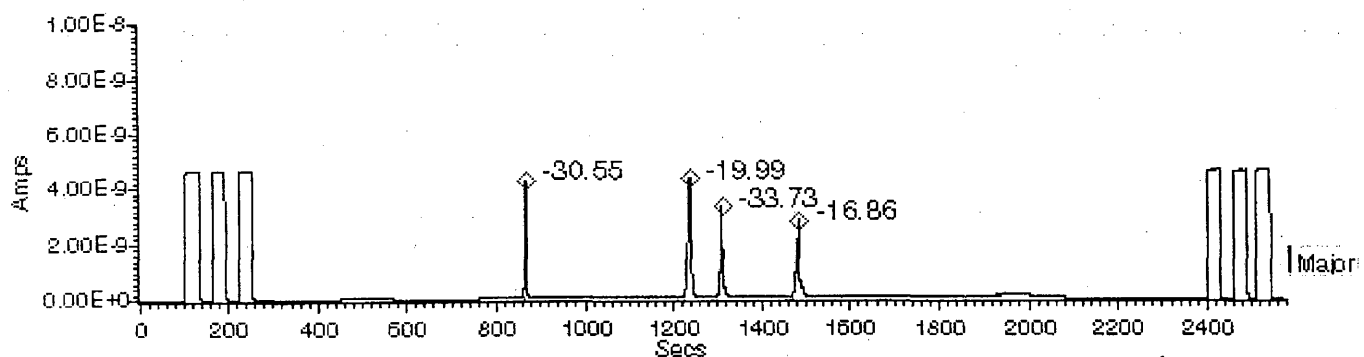
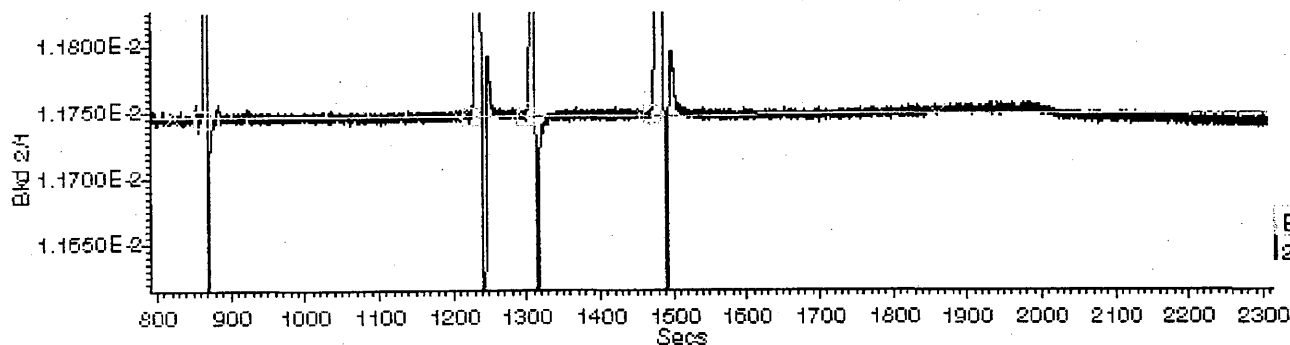
88

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_009 Folder : 040806
 Date : 04/08/06 Time : 12:24:14
 Comment : Mix Cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

× **Data Processing Main Graph** □ □

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

89

Data Processing Results

Data File Name : DATA_009
 Folder : 040806
 Sample Name : Mix Cal Acetate 001A-100ng inj
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 12:24:14 Date : 04/08/06
 Current Time : 09:52:20 Date : 05/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.507E-8 | 1.1776E-2 | 4.2528E-3 |
| 182.6 | 8.550E-8 | 1.1776E-2 | 4.2528E-3 |
| 242.6 | 8.493E-8 | 1.1776E-2 | 4.2530E-3 |
| 2423.4 | 8.422E-8 | 1.1775E-2 | 4.2523E-3 |
| 2483.5 | 8.354E-8 | 1.1775E-2 | 4.2518E-3 |
| 2533.5 | 8.443E-8 | 1.1775E-2 | 4.2521E-3 |

Std Dev Of Fit 9.2732E-8 1.7392E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 870.6 | 4.30E-9 | 1.9626E-8 | 1.1814E-2 | 4.1630E-3 | -30.44 | -76.57 | -39.98 | 32 |
| 1241.8 | 4.36E-9 | 3.6890E-8 | 1.1933E-2 | 4.1660E-3 | -20.05 | -74.35 | -39.29 | 31 |
| 1316.7 | 3.35E-9 | 2.8027E-8 | 1.1776E-2 | 4.1669E-3 | -33.81 | -73.88 | -39.05 | 31 |
| 1491.1 | 2.83E-9 | 3.4295E-8 | 1.1964E-2 | 4.1710E-3 | -17.36 | -72.44 | -38.14 | 31 |

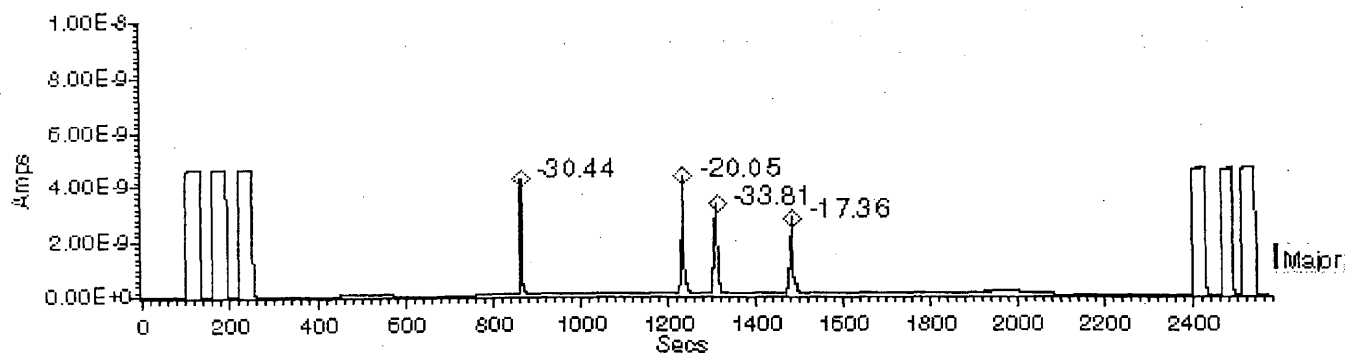
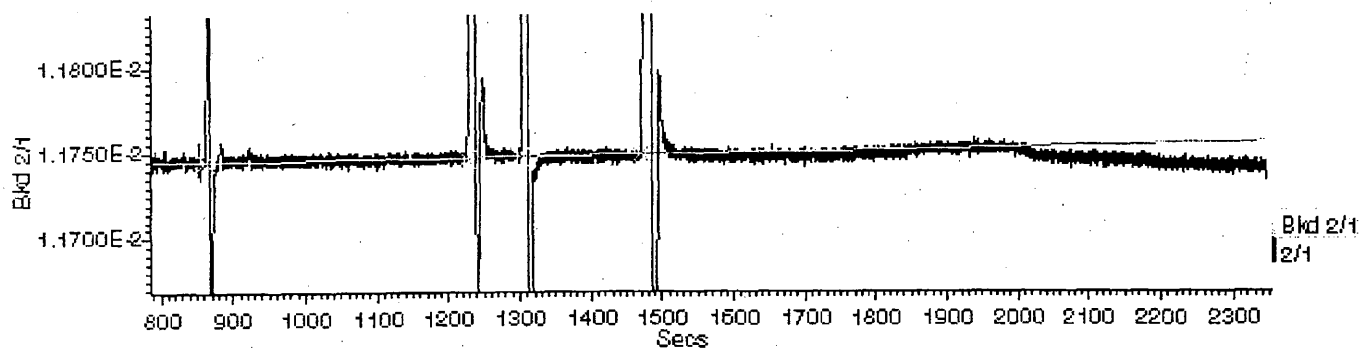
COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_009 Folder : 040806
 Date : 04/08/06 Time : 12:24:14
 Comment : Mix Cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

√ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Zero

Data File Name : DATA_009
 Folder : 040806
 Sample Name : Mix Cal Acetate 001A-100ng inj
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 12:24:14 Date : 04/08/06
 Current Time : 09:57:27 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.507E-8 | 1.1776E-2 | 4.2528E-3 |
| 182.6 | 8.550E-8 | 1.1776E-2 | 4.2528E-3 |
| 242.6 | 8.493E-8 | 1.1776E-2 | 4.2530E-3 |
| 2423.4 | 8.422E-8 | 1.1775E-2 | 4.2523E-3 |
| 2483.5 | 8.354E-8 | 1.1775E-2 | 4.2518E-3 |
| 2533.5 | 8.443E-8 | 1.1775E-2 | 4.2521E-3 |

Std Dev Of Fit 9.2732E-8 1.7392E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

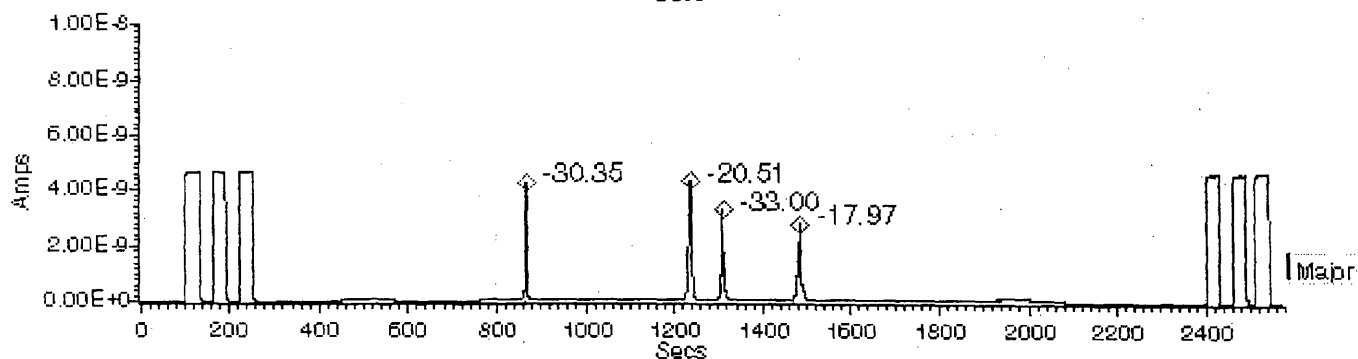
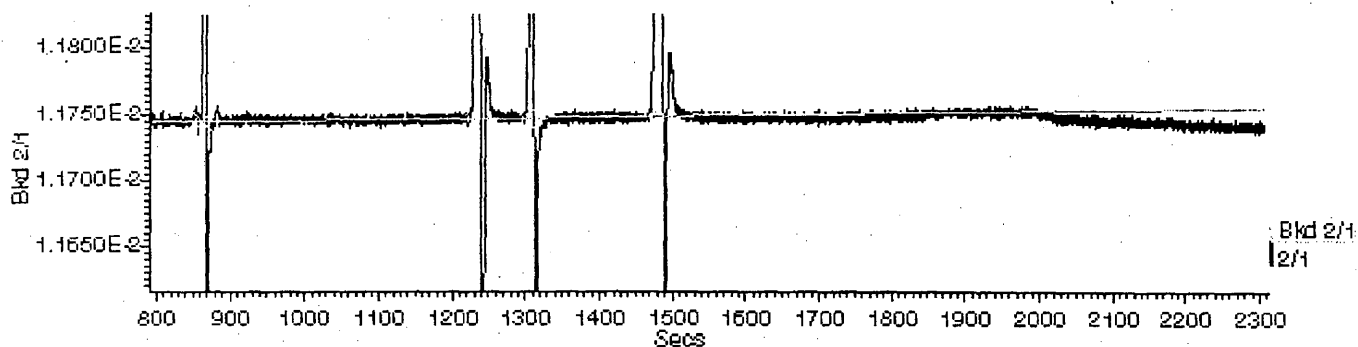
| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 870.6 | 4.30E-9 | 1.9626E-8 | 1.1816E-2 | 4.1648E-3 | -30.35 | -39.57 > |
| 1241.8 | 4.36E-9 | 3.6890E-8 | 1.1928E-2 | 4.1668E-3 | -20.51 | -39.11<> |
| 1316.7 | 3.35E-9 | 2.8027E-8 | 1.1785E-2 | 4.1666E-3 | -33.00 | -39.13<> |
| 1491.1 | 2.83E-9 | 3.4295E-8 | 1.1956E-2 | 4.1646E-3 | -17.97 | -39.60< |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

Data Filename : DATA_009 Folder : 040806
 Date : 04/08/06 Time : 12:24:14
 Comment : Mix Cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

93

Data Processing Results

Manuel

Data File Name : DATA_010
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F3/45uL inj 2uL
 Sample ID :
 Sample Position : 3
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 17:03:53 Date : 04/08/06
 Current Time : 10:05:57 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.545E-8 | 1.1774E-2 | 4.2514E-3 |
| 182.6 | 8.537E-8 | 1.1773E-2 | 4.2513E-3 |
| 242.7 | 8.497E-8 | 1.1773E-2 | 4.2507E-3 |
| 2423.5 | 8.468E-8 | 1.1772E-2 | 4.2505E-3 |
| 2483.5 | 8.400E-8 | 1.1772E-2 | 4.2507E-3 |
| 2533.5 | 8.435E-8 | 1.1772E-2 | 4.2508E-3 |

Std Dev Of Fit 3.7547E-7 2.7523E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 799.0 | 1.36E-8 | 5.8132E-8 | 1.1873E-2 | 4.1762E-3 | -25.17 | -60.99 | -36.59 | 17 |
| | 872.4 | 5.30E-9 | 2.2035E-8 | 1.1795E-2 | 4.1792E-3 | -31.98 | -61.55 | -35.89 | 17 |
| | 881.1 | 2.34E-9 | 1.3413E-8 | 1.1854E-2 | 4.2176E-3 | -27.15 | -61.55 | -27.03 | 17 |
| | 1260.3 | 2.49E-9 | 2.1552E-8 | 1.1840E-2 | 4.1927E-3 | -28.18 | -63.65 | -32.75 | 19 |
| | 1323.0 | 7.04E-9 | 6.5008E-8 | 1.1844E-2 | 4.1705E-3 | -27.63 | -63.93 | -37.87 | 20 |
| | 1353.8 | 1.98E-9 | 1.6167E-8 | 1.1834E-2 | 4.1883E-3 | -28.62 | -64.12 | -33.77 | 20 |
| | 1394.3 | 1.68E-9 | 1.5005E-8 | 1.1841E-2 | 4.1901E-3 | -28.02 | -64.28 | -33.34 | 20 |
| | 1674.1 | 3.26E-9 | 3.5570E-8 | 1.1855E-2 | 4.1632E-3 | -26.62 | -65.32 | -39.55 | 22 |

COPIE CERTIFIÉE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

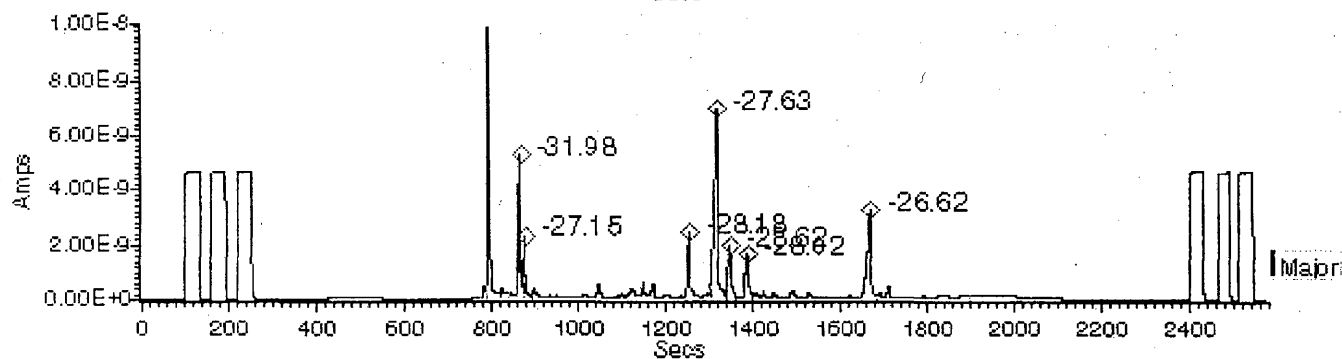
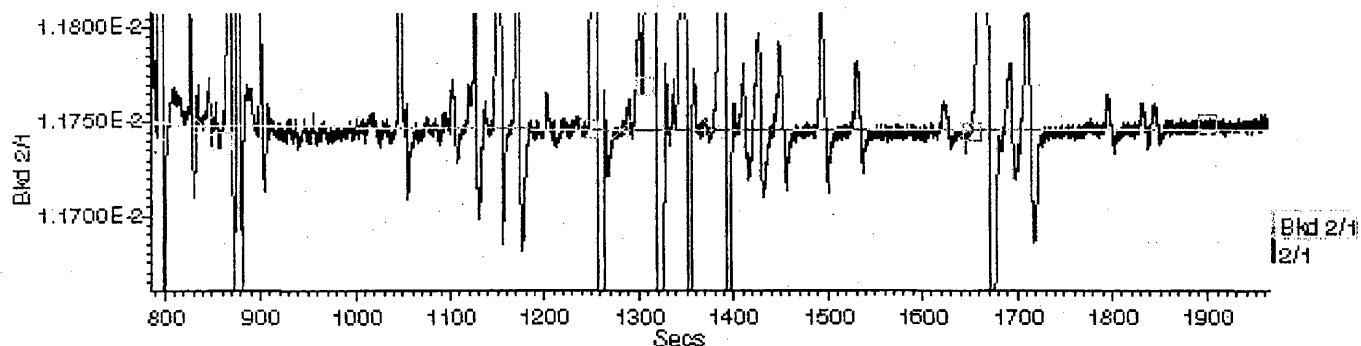
File Edit View Calculate Report Parameters Status Help

Data Filename: DATA_010 Folder: 040806
 Date: 04/08/06 Time: 17:03:53
 Comment: Blu 1 Pool 4 F3/45uL inj 2uL:
 Parameters Automatic DP Params

Data Processing Main Graph

101

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

95

Data Processing Results

Data File Name : DATA_010
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F3/45uL inj 2uL
 Sample ID :
 Sample Position : 3
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 17:03:53 Date : 04/08/06
 Current Time : 09:59:14 Date : 05/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.545E-8 | 1.1774E-2 | 4.2514E-3 |
| 182.6 | 8.537E-8 | 1.1773E-2 | 4.2513E-3 |
| 242.7 | 8.497E-8 | 1.1773E-2 | 4.2507E-3 |
| 2423.5 | 8.468E-8 | 1.1772E-2 | 4.2505E-3 |
| 2483.5 | 8.400E-8 | 1.1772E-2 | 4.2507E-3 |
| 2533.5 | 8.435E-8 | 1.1772E-2 | 4.2508E-3 |

Std Dev Of Fit 3.7547E-7 2.7523E-7

Analysis of Sample Peaks, with Background Subtraction

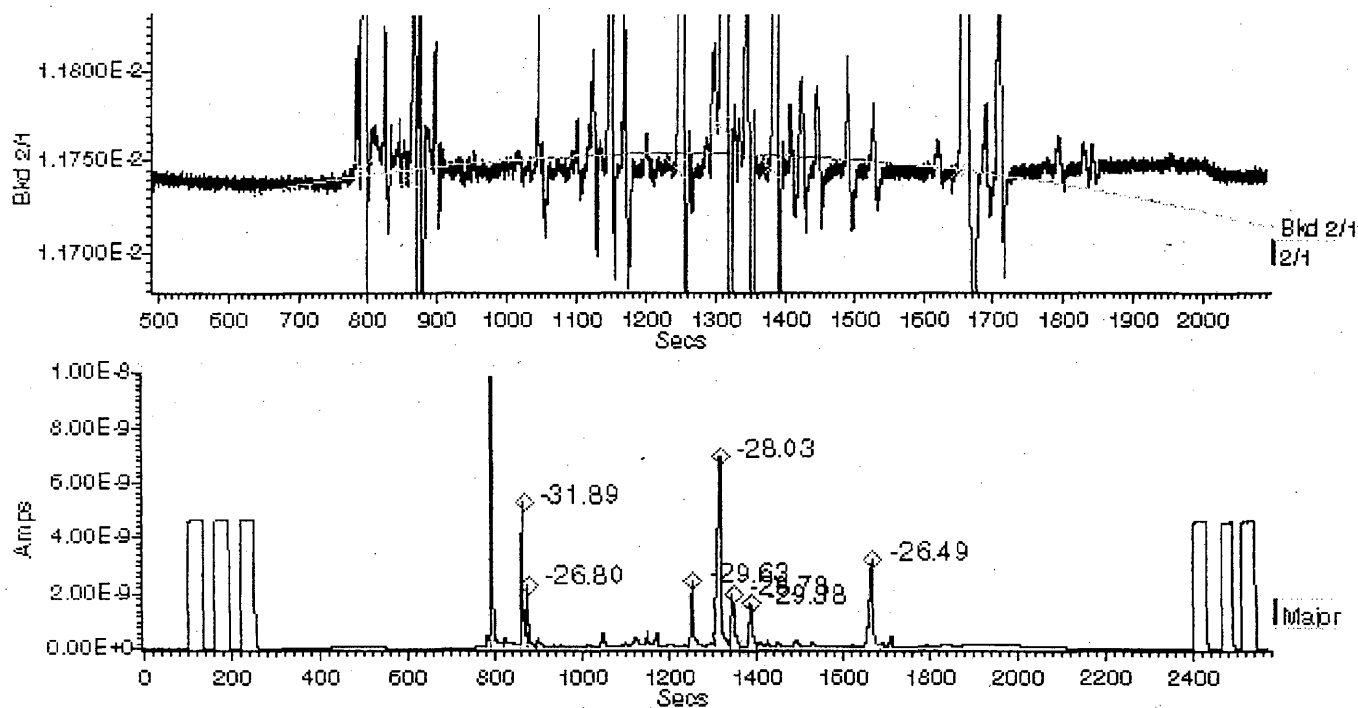
| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 799.0 | 1.36E-8 | 5.8132E-8 | 1.1876E-2 | 4.1762E-3 | -24.86 | -65.80 | -36.59 | 17 |
| | 872.4 | 5.30E-9 | 2.2765E-8 | 1.1797E-2 | 4.1852E-3 | -31.89 | -63.72 | -34.49 | 17 |
| | 881.1 | 2.34E-9 | 1.3413E-8 | 1.1858E-2 | 4.2176E-3 | -26.80 | -63.14 | -27.03 | 17 |
| | 1260.3 | 2.49E-9 | 2.2592E-8 | 1.1824E-2 | 4.1987E-3 | -29.63 | -57.82 | -31.37 | 19 |
| | 1323.0 | 7.04E-9 | 6.5008E-8 | 1.1839E-2 | 4.1705E-3 | -28.03 | -58.15 | -37.87 | 20 |
| | 1353.8 | 1.98E-9 | 1.6534E-8 | 1.1821E-2 | 4.1904E-3 | -29.78 | -58.47 | -33.27 | 20 |
| | 1394.3 | 1.68E-9 | 1.5157E-8 | 1.1826E-2 | 4.1913E-3 | -29.38 | -58.95 | -33.08 | 20 |
| | 1674.1 | 3.26E-9 | 3.5570E-8 | 1.1856E-2 | 4.1632E-3 | -26.49 | -66.31 | -39.55 | 22 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Filename : DATA_010 Folder : 040806
 Date : 04/08/06 Time : 17:03:53
 Comment : Blu 1 Pool 4 F3/45uL inj 2uL :
 Parameters Automatic DP Params

∨ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

97

Data Processing Results

Zero .

Data File Name : DATA_010
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F3/45uL inj 2uL
 Sample ID :
 Sample Position : 3
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 17:03:53 Date : 04/08/06
 Current Time : 10:07:15 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.545E-8 | 1.1774E-2 | 4.2514E-3 |
| 182.6 | 8.537E-8 | 1.1773E-2 | 4.2513E-3 |
| 242.7 | 8.497E-8 | 1.1773E-2 | 4.2507E-3 |
| 2423.5 | 8.468E-8 | 1.1772E-2 | 4.2505E-3 |
| 2483.5 | 8.400E-8 | 1.1772E-2 | 4.2507E-3 |
| 2533.5 | 8.435E-8 | 1.1772E-2 | 4.2508E-3 |

Std Dev Of Fit 3.7547E-7 2.7523E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 799.0 | 1.36E-8 | 5.8132E-8 | 1.1874E-2 | 4.1607E-3 | -24.96 | -40.17 > |
| 872.4 | 5.30E-9 | 2.2765E-8 | 1.1801E-2 | 4.1636E-3 | -31.39 | -39.49<> |
| 881.1 | 2.34E-9 | 1.3413E-8 | 1.1859E-2 | 4.1664E-3 | -26.32 | -38.85<> |
| 1260.3 | 2.49E-9 | 2.2592E-8 | 1.1843E-2 | 4.1654E-3 | -27.70 | -39.05<> |
| 1323.0 | 7.04E-9 | 6.5008E-8 | 1.1845E-2 | 4.1618E-3 | -27.48 | -39.88<> |
| 1353.8 | 1.98E-9 | 1.6534E-8 | 1.1839E-2 | 4.1649E-3 | -28.06 | -39.16<> |
| 1394.3 | 1.68E-9 | 1.5157E-8 | 1.1844E-2 | 4.1641E-3 | -27.55 | -39.35<> |
| 1674.1 | 3.26E-9 | 3.5570E-8 | 1.1855E-2 | 4.1620E-3 | -26.60 | -39.83< |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

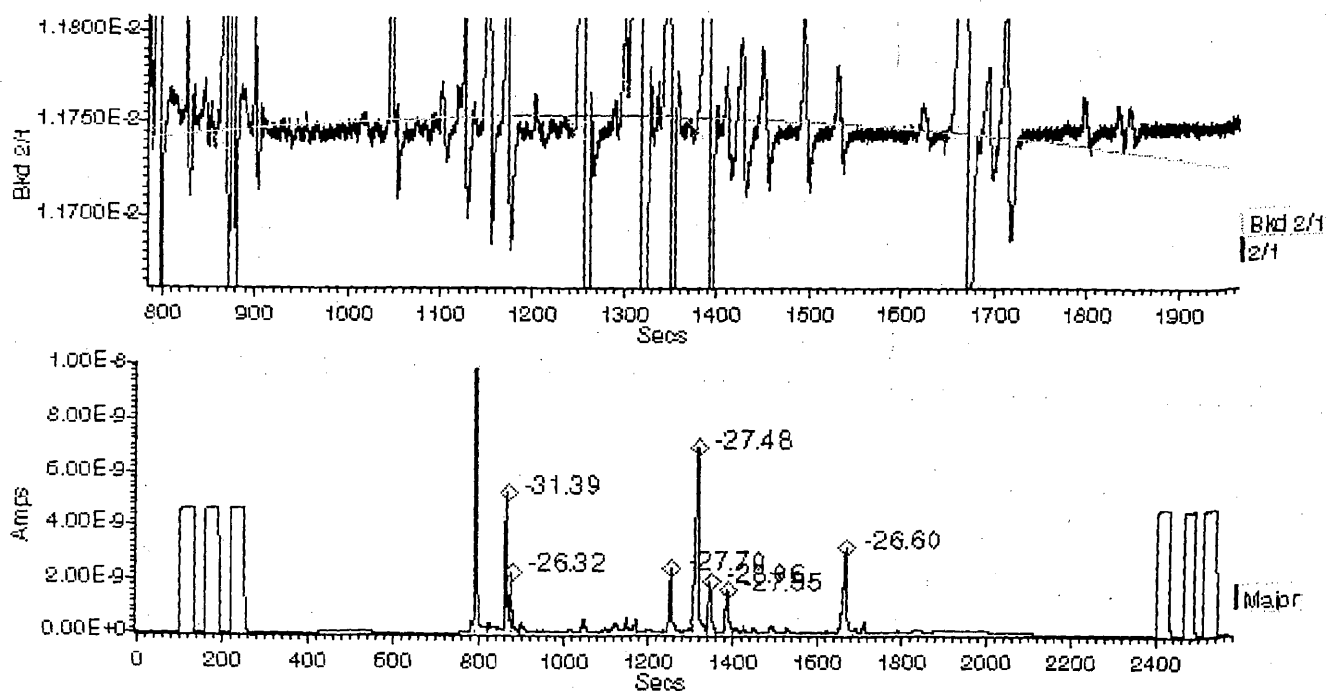
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_010 Folder : 040806
 Date : 04/06/06 Time : 17:03:53
 Comment : Blu 1 Pool 4 F3/45uL inj 2uL :
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

99

Data Processing Results

Data File Name : DATA_011
 Folder : 040806
 Sample Name : 178/07 995474 F3/45uL inj 2uL
 Sample ID :
 Sample Position : 4
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 17:48:39 Date : 04/08/06
 Current Time : 10:19:31 Date : 05/05/07

Manual

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.7 | 8.465E-8 | 1.1772E-2 | 4.2501E-3 |
| 182.7 | 8.465E-8 | 1.1771E-2 | 4.2504E-3 |
| 242.7 | 8.459E-8 | 1.1771E-2 | 4.2502E-3 |
| 2423.5 | 8.450E-8 | 1.1772E-2 | 4.2501E-3 |
| 2483.6 | 8.436E-8 | 1.1772E-2 | 4.2501E-3 |
| 2533.5 | 8.541E-8 | 1.1772E-2 | 4.2506E-3 |

Std Dev Of Fit 3.1407E-7 2.2560E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 797.1 | 1.84E-9 | 1.7724E-8 | 1.1816E-2 | 4.2650E-3 | -30.70 | -50.75 | -15.90 | 8 |
| 839.1 | 1.63E-9 | 9.1742E-9 | 1.1752E-2 | 4.2295E-3 | -36.07 | -51.20 | -24.09 | 9 |
| 871.9 | 4.08E-9 | 1.9175E-8 | 1.1808E-2 | 4.1891E-3 | -30.83 | -51.44 | -33.42 | 9 |
| 880.6 | 4.07E-9 | 2.2454E-8 | 1.1827E-2 | 4.1969E-3 | -29.26 | -51.50 | -31.63 | 9 |
| 909.0 | 3.27E-9 | 2.5928E-8 | 1.1760E-2 | 4.2250E-3 | -35.35 | -51.65 | -25.14 | 9 |
| 1318.2 | 4.22E-9 | 3.8729E-8 | 1.1824E-2 | 4.1753E-3 | -29.36 | -53.87 | -36.62 | 12 |
| 1352.4 | 2.33E-9 | 2.0473E-8 | 1.1785E-2 | 4.1836E-3 | -32.78 | -54.00 | -34.69 | 12 |
| 1671.2 | 2.57E-9 | 2.8872E-8 | 1.1856E-2 | 4.1589E-3 | -26.40 | -54.46 | -40.43 | 14 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

100
 GDC00969

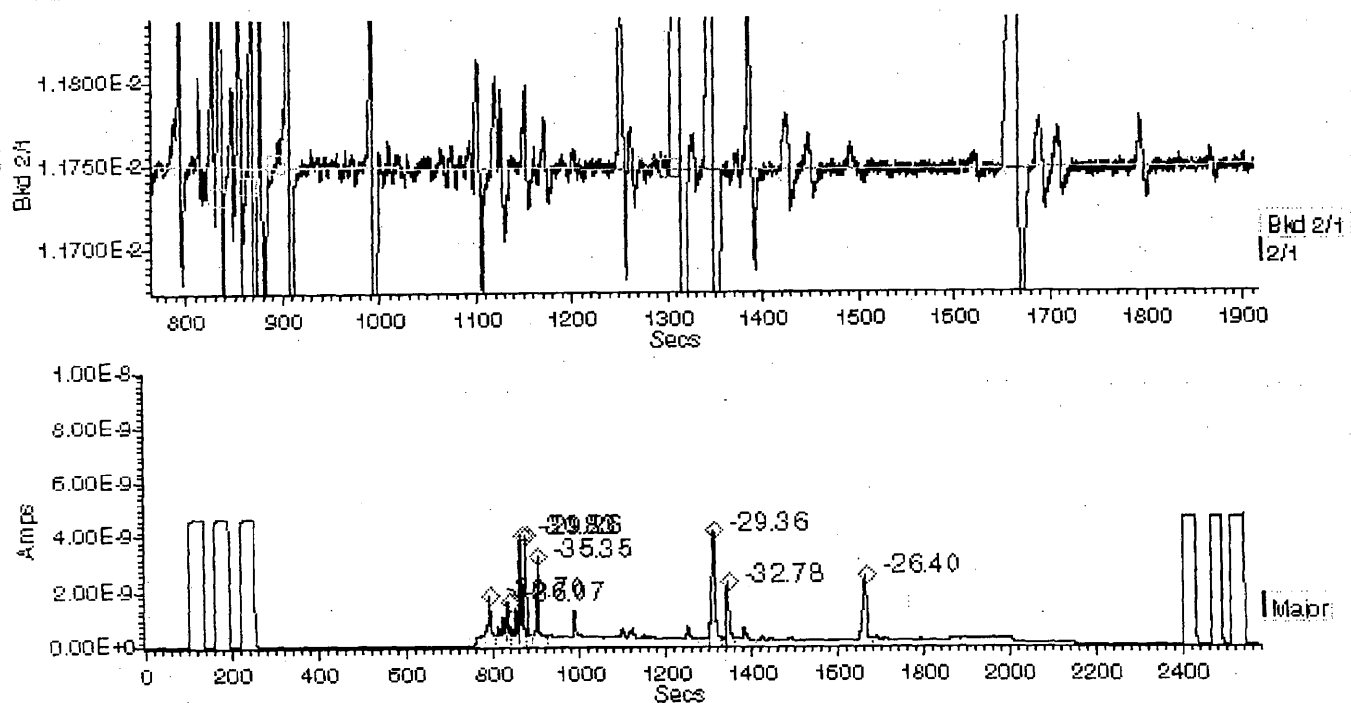
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename: DATA_011 Folder : 040806
 Date : 04/08/06 Time : 17:48:39
 Comment : 178/07 995474 F3/45uL inj 2uL :
 Parameters Automatic DP Params

× Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

101

Data Processing Results

Data File Name : DATA_011
 Folder : 040806
 Sample Name : 178/07 995474 F3/45uL inj 2uL
 Sample ID :
 Sample Position : 4
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 17:48:39 Date : 04/08/06
 Current Time : 10:09:47 Date : 05/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.7 | 8.465E-8 | 1.1772E-2 | 4.2501E-3 |
| 182.7 | 8.465E-8 | 1.1771E-2 | 4.2504E-3 |
| 242.7 | 8.459E-8 | 1.1771E-2 | 4.2502E-3 |
| 2423.5 | 8.450E-8 | 1.1772E-2 | 4.2501E-3 |
| 2483.6 | 8.436E-8 | 1.1772E-2 | 4.2501E-3 |
| 2533.5 | 8.541E-8 | 1.1772E-2 | 4.2506E-3 |

Std Dev Of Fit 3.1407E-7 2.2560E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | d018Pk | d018 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 797.1 | 1.84E-9 | 1.7724E-8 | 1.1836E-2 | 4.2650E-3 | -28.95 | -55.07 | -15.90 | 8 |
| 839.1 | 1.63E-9 | 9.1742E-9 | 1.1764E-2 | 4.2295E-3 | -35.03 | -54.92 | -24.09 | 9 |
| 871.9 | 4.08E-9 | 1.9175E-8 | 1.1813E-2 | 4.1891E-3 | -30.44 | -54.76 | -33.42 | 9 |
| 880.6 | 4.07E-9 | 2.2454E-8 | 1.1832E-2 | 4.1969E-3 | -28.76 | -54.64 | -31.63 | 9 |
| 909.0 | 3.27E-9 | 2.5928E-8 | 1.1769E-2 | 4.2250E-3 | -34.51 | -54.46 | -25.14 | 9 |
| 1318.2 | 4.22E-9 | 3.9586E-8 | 1.1824E-2 | 4.1764E-3 | -29.37 | -53.92 | -36.37 | 12 |
| 1352.4 | 2.33E-9 | 2.1502E-8 | 1.1785E-2 | 4.1861E-3 | -32.87 | -54.00 | -34.13 | 12 |
| 1671.2 | 2.57E-9 | 2.8872E-8 | 1.1859E-2 | 4.1589E-3 | -26.14 | -55.40 | -40.43 | 14 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

GDC00971

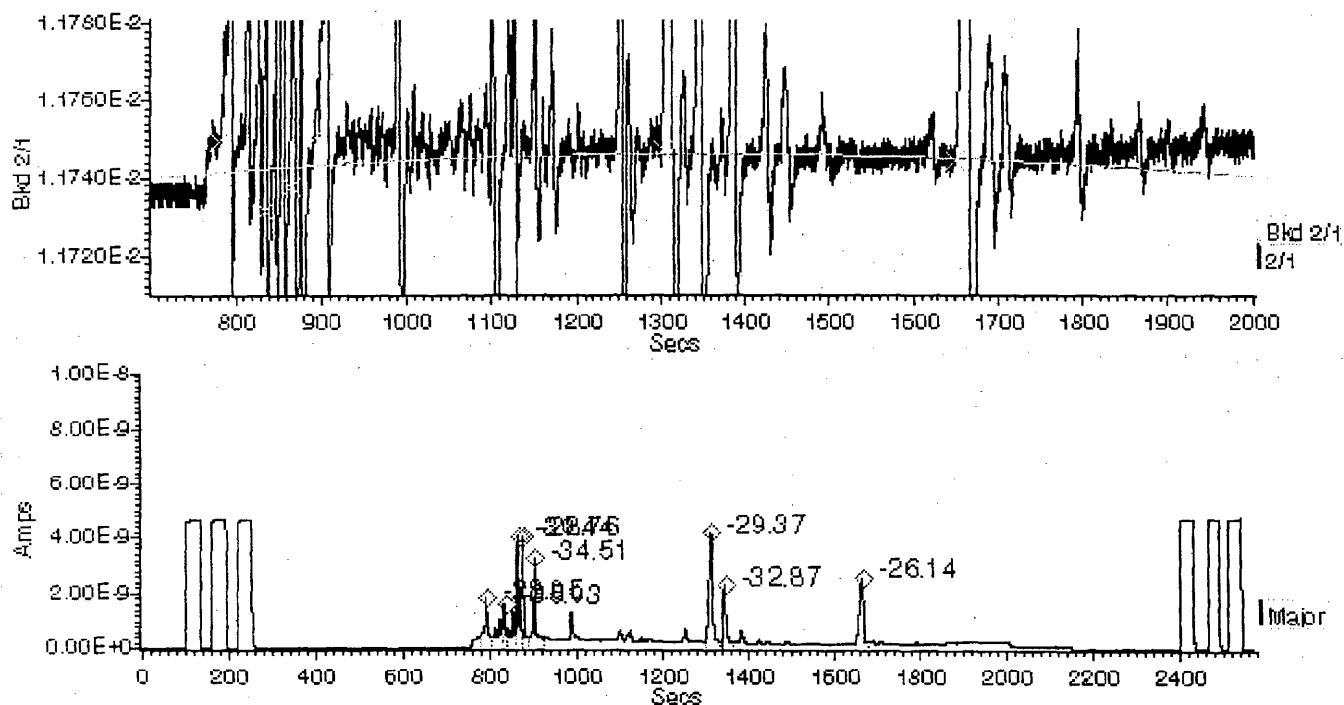
102

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_011 Folder : 040806
Date : 04/08/06 Time : 17:48:39
Comment : 178/07 995474 F3/45uL inj 2uL
Parameters Automatic DP Params

Data Processing Main Graph

□ □

Graph Cursor Lines Window

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

103

Data Processing Results

Data File Name : DATA_011
 Folder : 040806
 Sample Name : 178/07 995474 F3/45uL inj 2uL
 Sample ID :
 Sample Position : 4
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 17:48:39 Date : 04/08/06
 Current Time : 10:21:27 Date : 05/05/07

Zero

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.7 | 8.465E-8 | 1.1772E-2 | 4.2501E-3 |
| 182.7 | 8.465E-8 | 1.1771E-2 | 4.2504E-3 |
| 242.7 | 8.459E-8 | 1.1771E-2 | 4.2502E-3 |
| 2423.5 | 8.450E-8 | 1.1772E-2 | 4.2501E-3 |
| 2483.6 | 8.436E-8 | 1.1772E-2 | 4.2501E-3 |
| 2533.5 | 8.541E-8 | 1.1772E-2 | 4.2506E-3 |

Std Dev Of Fit 3.1407E-7 2.2560E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 797.1 | 1.84E-9 | 1.7724E-8 | 1.1826E-2 | 4.1695E-3 | -29.07 | -37.96 > |
| 839.1 | 1.63E-9 | 9.1742E-9 | 1.1771E-2 | 4.1675E-3 | -33.94 | -38.41<> |
| 871.9 | 4.08E-9 | 1.9175E-8 | 1.1812E-2 | 4.1650E-3 | -30.31 | -39.01<> |
| 880.6 | 4.07E-9 | 2.2454E-8 | 1.1829E-2 | 4.1648E-3 | -28.77 | -39.05<> |
| 909.0 | 3.27E-9 | 2.5928E-8 | 1.1777E-2 | 4.1664E-3 | -33.39 | -38.67<> |
| 1318.2 | 4.22E-9 | 3.9586E-8 | 1.1824E-2 | 4.1641E-3 | -29.22 | -39.21<> |
| 1352.4 | 2.33E-9 | 2.1502E-8 | 1.1794E-2 | 4.1662E-3 | -31.90 | -38.72<> |
| 1671.2 | 2.57E-9 | 2.8872E-8 | 1.1851E-2 | 4.1654E-3 | -26.86 | -38.92< |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

GDC00973

104

DP Optima GC 1.67-2 - Manual DP

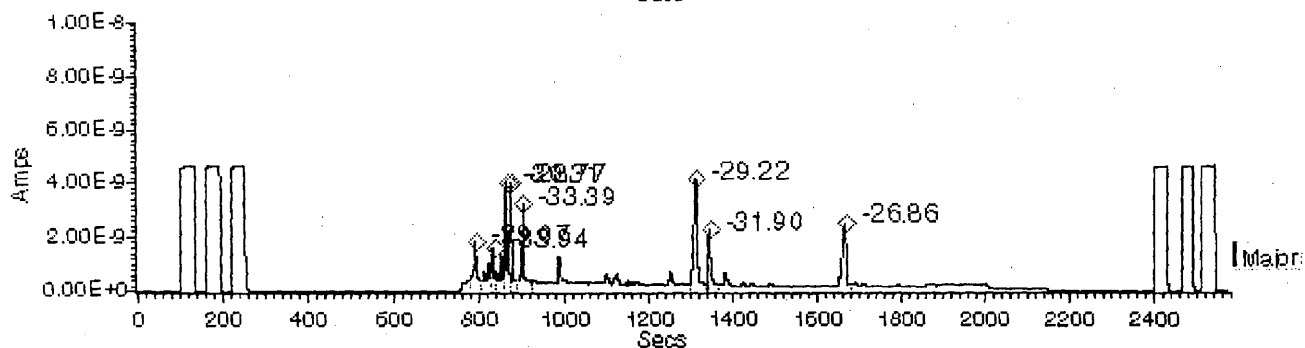
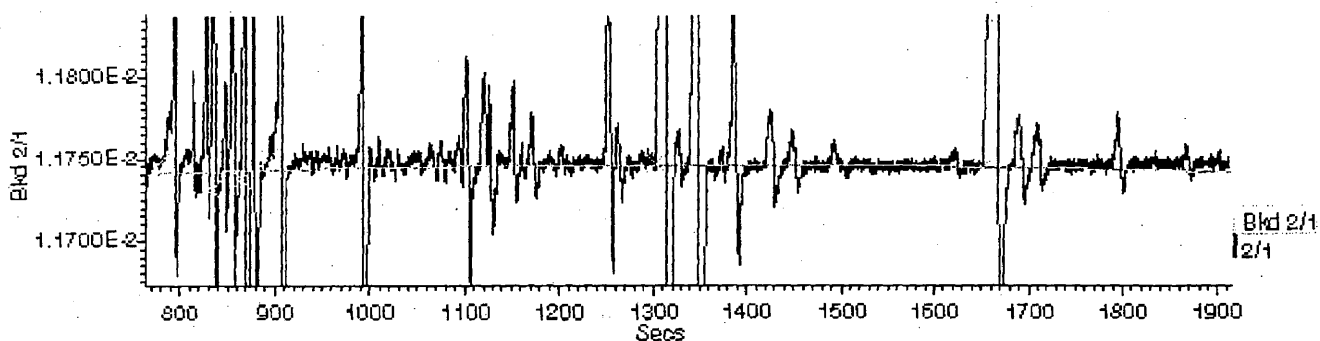
File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_011 Folder : 040806
Date : 04/08/06 Time : 17:48:39
Comment : 178/07 995474 F3/45uL inj 2uL :

Parameters Automatic DP Params

≡ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

105

Data Processing Results

Data File Name : DATA_012
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F1/120uL inj 2uL
 Sample ID :
 Sample Position : 5
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 18:33:24 Date : 04/08/06
 Current Time : 10:27:24 Date : 05/05/07

Manual.

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.532E-8 | 1.1773E-2 | 4.2515E-3 |
| 182.6 | 8.541E-8 | 1.1773E-2 | 4.2517E-3 |
| 242.6 | 8.493E-8 | 1.1773E-2 | 4.2515E-3 |
| 2423.5 | 8.481E-8 | 1.1773E-2 | 4.2516E-3 |
| 2483.5 | 8.479E-8 | 1.1773E-2 | 4.2516E-3 |
| 2533.5 | 8.517E-8 | 1.1773E-2 | 4.2520E-3 |

Std Dev Of Fit 1.3906E-7 1.7907E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

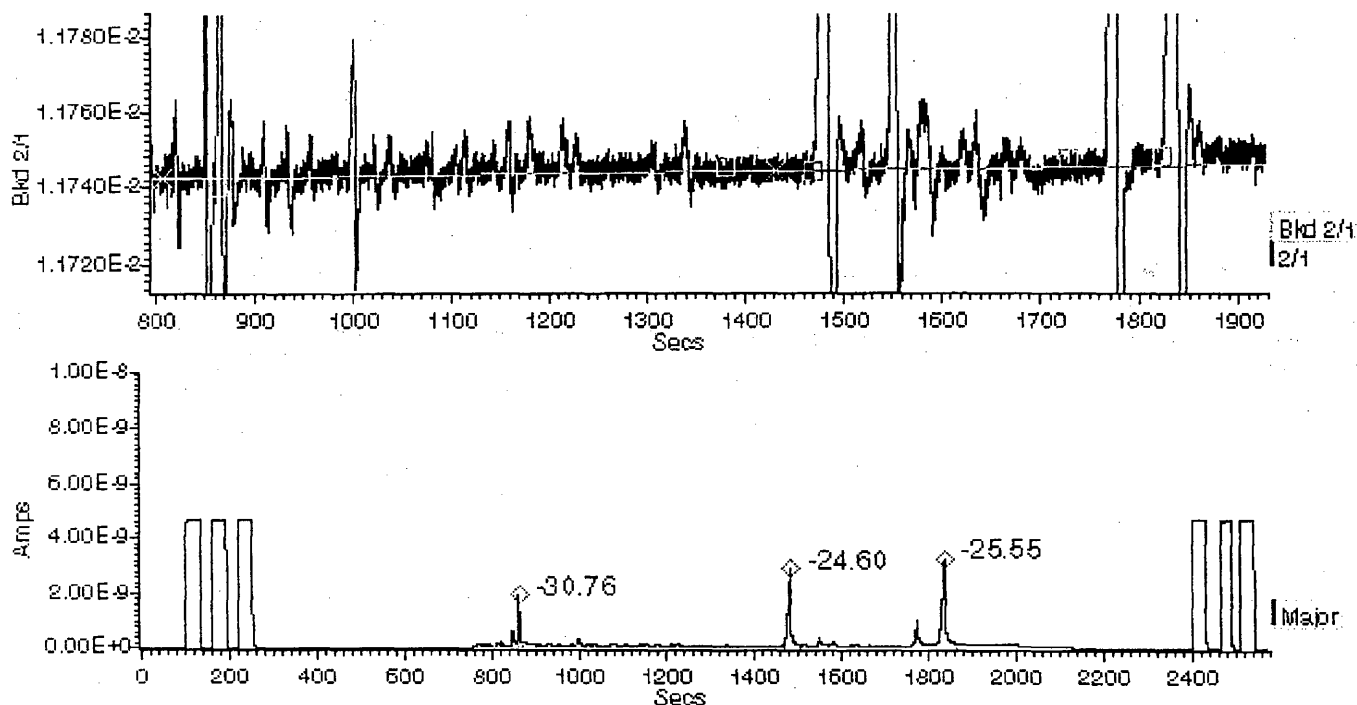
| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 869.6 | 1.97E-9 | 9.0004E-9 | 1.1810E-2 | 4.1840E-3 | -30.76 | -62.77 | -34.91 | 17 |
| 1489.8 | 2.93E-9 | 3.0009E-8 | 1.1878E-2 | 4.1636E-3 | -24.60 | -61.98 | -39.65 | 19 |
| 1843.5 | 3.29E-9 | 3.8707E-8 | 1.1868E-2 | 4.1724E-3 | -25.55 | -60.76 | -37.63 | 18 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Filename : DATA_012 Folder : 040806
 Date : 04/08/06 Time : 18:33:24
 Comment : Blu 1 Pool 4 F1/120uL inj 2uL :
 Parameters Automatic DP Params

√ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

107

Data Processing Results

Data File Name : DATA_012
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F1/120uL inj 2uL
 Sample ID :
 Sample Position : 5
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 18:33:24 Date : 04/08/06
 Current Time : 10:23:10 Date : 05/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.532E-8 | 1.1773E-2 | 4.2515E-3 |
| 182.6 | 8.541E-8 | 1.1773E-2 | 4.2517E-3 |
| 242.6 | 8.493E-8 | 1.1773E-2 | 4.2515E-3 |
| 2423.5 | 8.481E-8 | 1.1773E-2 | 4.2516E-3 |
| 2483.5 | 8.479E-8 | 1.1773E-2 | 4.2516E-3 |
| 2533.5 | 8.517E-8 | 1.1773E-2 | 4.2520E-3 |

Std Dev Of Fit 1.3906E-7 1.7907E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 869.6 | 1.97E-9 | 9.0004E-9 | 1.1813E-2 | 4.1841E-3 | -30.45 | -64.35 | -34.89 | 18 |
| | 1489.8 | 2.93E-9 | 3.0458E-8 | 1.1879E-2 | 4.1636E-3 | -24.56 | -62.12 | -39.64 | 20 |
| | 1843.5 | 3.29E-9 | 3.8707E-8 | 1.1864E-2 | 4.1725E-3 | -25.95 | -58.90 | -37.61 | 18 |

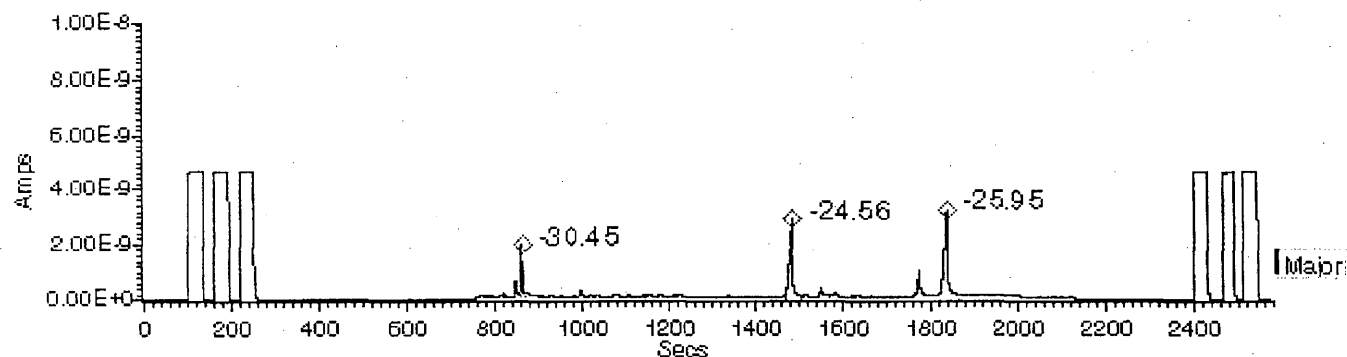
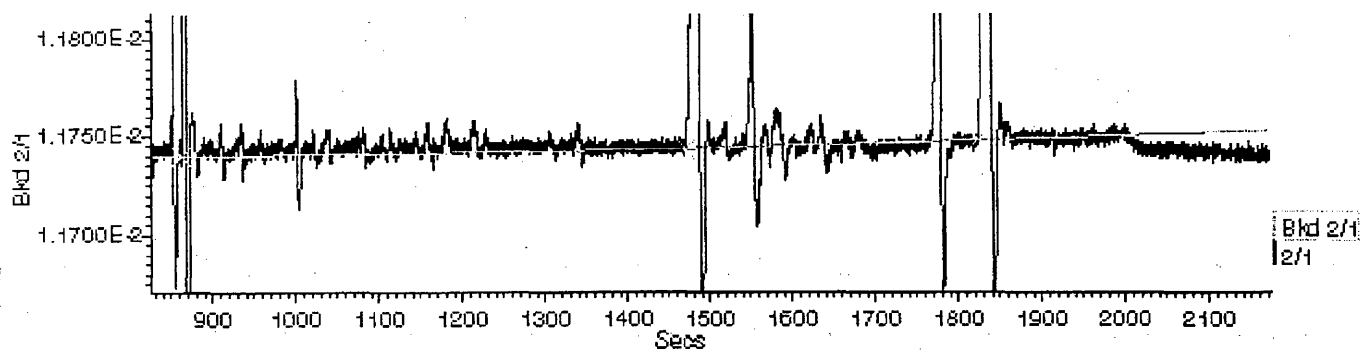
COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

108
 GDC00977

Data Filename : DATA_012 Folder : 040806
 Date : 04/08/06 Time : 18:33:24
 Comment : Blu 1 Pool 4 F1/120uL inj 2uL :
 Parameters Automatic DP Params

▾ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

109

Data Processing Results

Data File Name : DATA_012
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F1/120uL inj 2uL
 Sample ID :
 Sample Position : 5
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 18:33:24 Date : 04/08/06
 Current Time : 10:28:29 Date : 05/05/07

Zero

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.532E-8 | 1.1773E-2 | 4.2515E-3 |
| 182.6 | 8.541E-8 | 1.1773E-2 | 4.2517E-3 |
| 242.6 | 8.493E-8 | 1.1773E-2 | 4.2515E-3 |
| 2423.5 | 8.481E-8 | 1.1773E-2 | 4.2516E-3 |
| 2483.5 | 8.479E-8 | 1.1773E-2 | 4.2516E-3 |
| 2533.5 | 8.517E-8 | 1.1773E-2 | 4.2520E-3 |

Std Dev Of Fit 1.3906E-7 1.7907E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 869.6 | 1.97E-9 | 9.0004E-9 | 1.1808E-2 | 4.1646E-3 | -30.75 | -39.39 > |
| 1489.8 | 2.93E-9 | 3.0458E-8 | 1.1871E-2 | 4.1639E-3 | -25.26 | -39.59<> |
| 1843.5 | 3.29E-9 | 3.8707E-8 | 1.1863E-2 | 4.1622E-3 | -25.91 | -39.98< |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

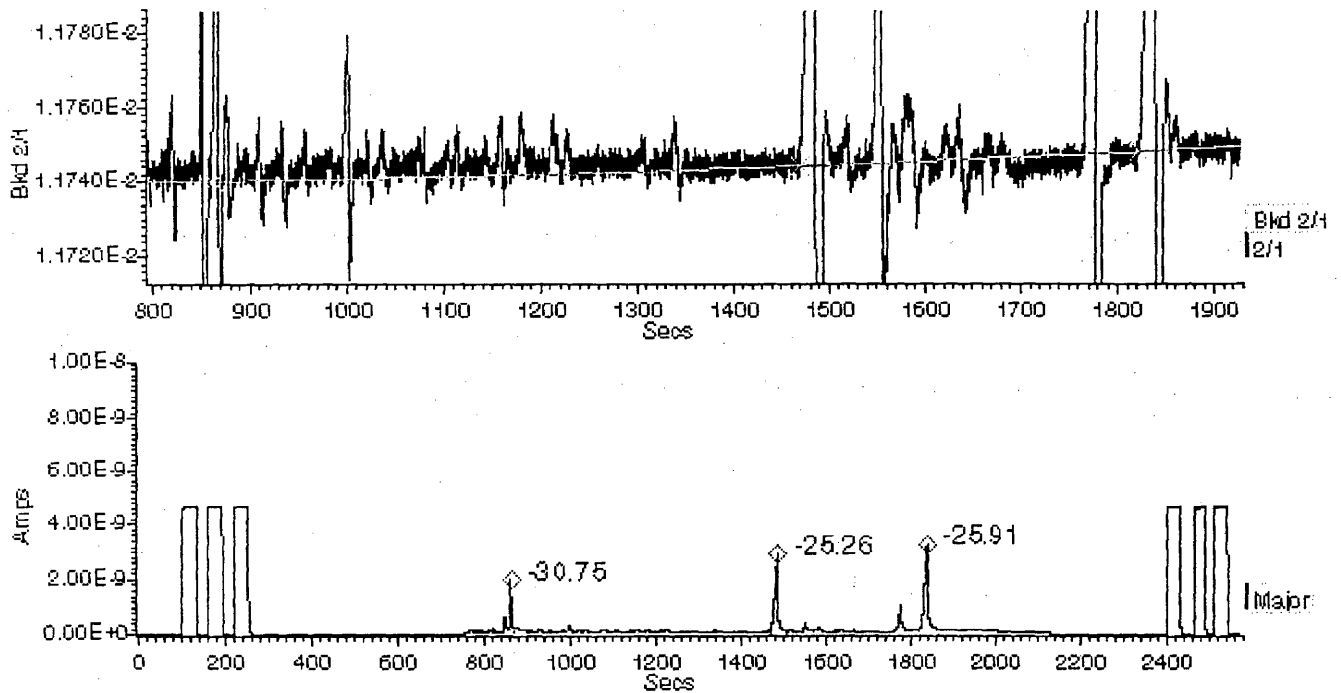
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_012 Folder : 040806
Date : 04/08/06 Time : 18:33:24
Comment : Blu 1 Pool 4 F1/120uL inj 2uL :
Parameters Automatic DP Params

√ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

M

Data Processing Results

Data File Name : DATA_013
 Folder : 040806
 Sample Name : 178/07 995474 F1/150uL inj 2uL
 Sample ID :
 Sample Position : 6
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 19:18:09 Date : 04/08/06
 Current Time : 10:36:34 Date : 05/05/07

Manual

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.496E-8 | 1.1774E-2 | 4.2514E-3 |
| 182.6 | 8.501E-8 | 1.1773E-2 | 4.2514E-3 |
| 242.7 | 8.541E-8 | 1.1773E-2 | 4.2517E-3 |
| 2423.5 | 8.495E-8 | 1.1775E-2 | 4.2521E-3 |
| 2483.5 | 8.456E-8 | 1.1775E-2 | 4.2522E-3 |
| 2533.5 | 8.598E-8 | 1.1775E-2 | 4.2528E-3 |

Std Dev Of Fit 3.6976E-7 2.8218E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 777.1 | 4.35E-9 | 2.3077E-8 | 1.1767E-2 | 4.1710E-3 | -34.48 | -111.76 | -37.94 | 63 |
| 870.2 | 2.90E-9 | 1.4883E-8 | 1.1798E-2 | 4.2137E-3 | -32.04 | -104.39 | -28.08 | 42 |
| 1490.1 | 2.72E-9 | 2.8911E-8 | 1.1864E-2 | 4.1863E-3 | -26.14 | -105.23 | -34.49 | 58 |

COPIE CERTIFIÉE
 CONFORME DES MAXIMA
 ET FORMULAIRES ORIGINAUX

GDC00981

112

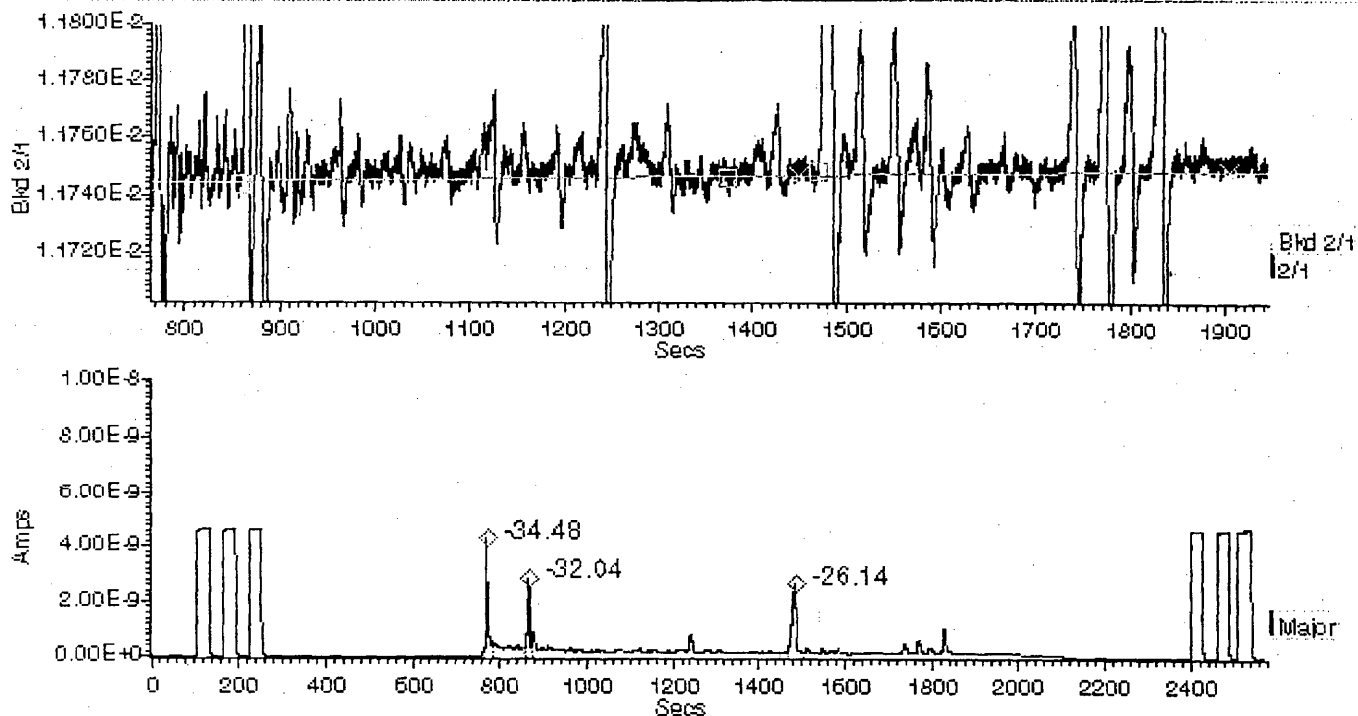
File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_013 Folder : 040806
Date : 04/08/06 Time : 19:18:09
Comment : 178/07 995474 F1/150uL inj 2uL

Parameters Automatic DP Params

√ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

113

Data Processing Results

Data File Name : DATA_013
 Folder : 040806
 Sample Name : 178/07 995474 F1/150uL inj 2uL
 Sample ID :
 Sample Position : 6
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 19:18:09 Date : 04/08/06
 Current Time : 10:29:58 Date : 05/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.496E-8 | 1.1774E-2 | 4.2514E-3 |
| 182.6 | 8.501E-8 | 1.1773E-2 | 4.2514E-3 |
| 242.7 | 8.541E-8 | 1.1773E-2 | 4.2517E-3 |
| 2423.5 | 8.495E-8 | 1.1775E-2 | 4.2521E-3 |
| 2483.5 | 8.456E-8 | 1.1775E-2 | 4.2522E-3 |
| 2533.5 | 8.598E-8 | 1.1775E-2 | 4.2528E-3 |

Std Dev Of Fit 3.6976E-7 2.8218E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 777.1 | 4.35E-9 | 2.3077E-8 | 1.1772E-2 | 4.1710E-3 | -34.00 | -121.78 | -37.94 | 63 |
| | 870.2 | 2.90E-9 | 1.5361E-8 | 1.1799E-2 | 4.2176E-3 | -32.03 | -106.50 | -27.19 | 43 |
| | 1490.1 | 2.72E-9 | 2.8911E-8 | 1.1863E-2 | 4.1863E-3 | -26.18 | -104.58 | -34.49 | 58 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

GDC00983

114

DP Optima GC 1.67-2 - Manual DP

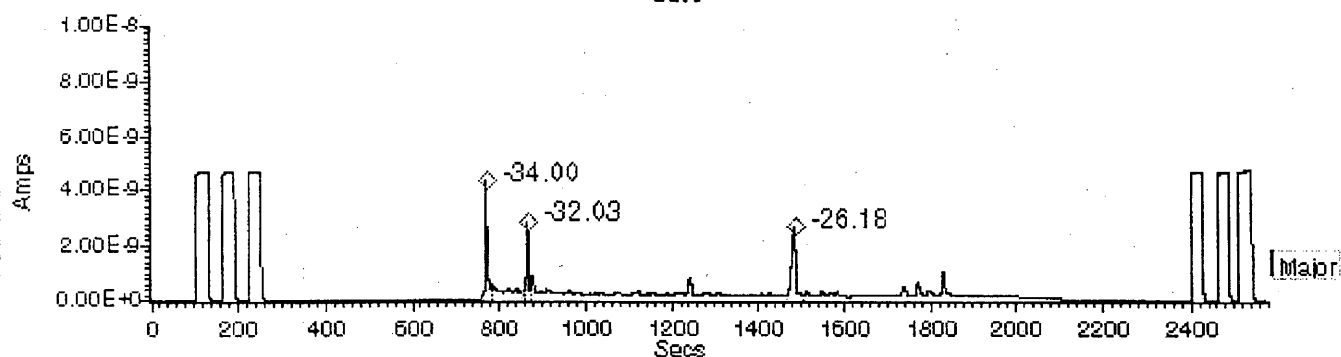
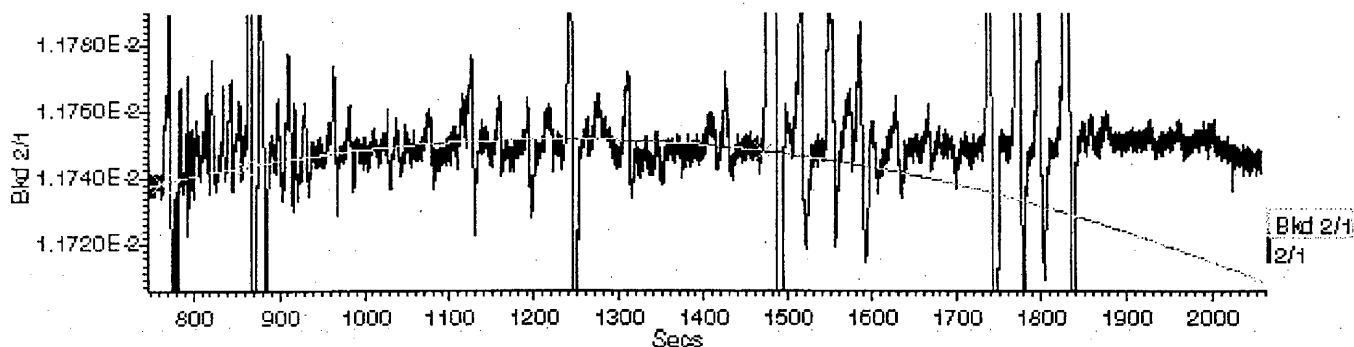
File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_013 Folder : 040806
 Date : 04/08/06 Time : 19:18:09
 Comment : 178/07 995474 F1/150uL inj 2uL

Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

115

Data Processing Results

Data File Name : DATA_013
 Folder : 040806
 Sample Name : 178/07 995474 F1/150uL inj 2uL
 Sample ID :
 Sample Position : 6
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 19:18:09 Date : 04/08/06
 Current Time : 10:37:24 Date : 05/05/07

Zero.

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.496E-8 | 1.1774E-2 | 4.2514E-3 |
| 182.6 | 8.501E-8 | 1.1773E-2 | 4.2514E-3 |
| 242.7 | 8.541E-8 | 1.1773E-2 | 4.2517E-3 |
| 2423.5 | 8.495E-8 | 1.1775E-2 | 4.2521E-3 |
| 2483.5 | 8.456E-8 | 1.1775E-2 | 4.2522E-3 |
| 2533.5 | 8.598E-8 | 1.1775E-2 | 4.2528E-3 |

Std Dev Of Fit 3.6976E-7 2.8218E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 777.1 | 4.35E-9 | 2.3077E-8 | 1.1774E-2 | 4.1661E-3 | -33.83 | -39.06 > |
| 870.2 | 2.90E-9 | 1.5361E-8 | 1.1805E-2 | 4.1655E-3 | -31.06 | -39.23<> |
| 1490.1 | 2.72E-9 | 2.8911E-8 | 1.1874E-2 | 4.1660E-3 | -25.08 | -39.18< |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

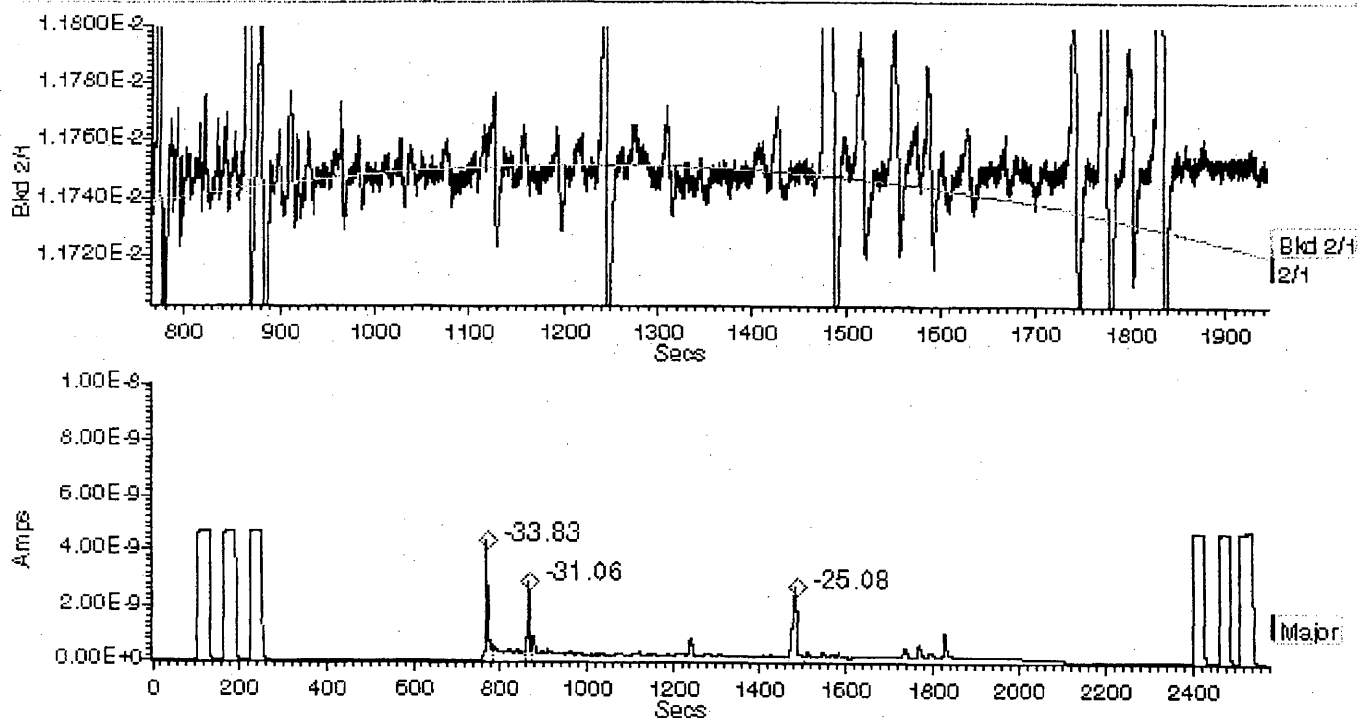
File Edit View Calculate Report Parameters Status Help

Data Filename: DATA_013 Folder: 040806
 Date: 04/08/06 Time: 19:18:09
 Comment: 178/07 995474 F1/150uL inj 2uL

Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

117

Data Processing Results

Data File Name : DATA_014
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F2/1400uL inj 2uL
 Sample ID :
 Sample Position : 7
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 20:02:53 Date : 04/08/06
 Current Time : 10:42:30 Date : 05/05/07

Manual.

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.496E-8 | 1.1776E-2 | 4.2531E-3 |
| 182.6 | 8.503E-8 | 1.1776E-2 | 4.2531E-3 |
| 242.6 | 8.494E-8 | 1.1776E-2 | 4.2528E-3 |
| 2423.5 | 8.442E-8 | 1.1774E-2 | 4.2519E-3 |
| 2483.5 | 8.385E-8 | 1.1774E-2 | 4.2515E-3 |
| 2533.5 | 8.462E-8 | 1.1773E-2 | 4.2518E-3 |

Std Dev Of Fit 3.6403E-7 1.7354E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

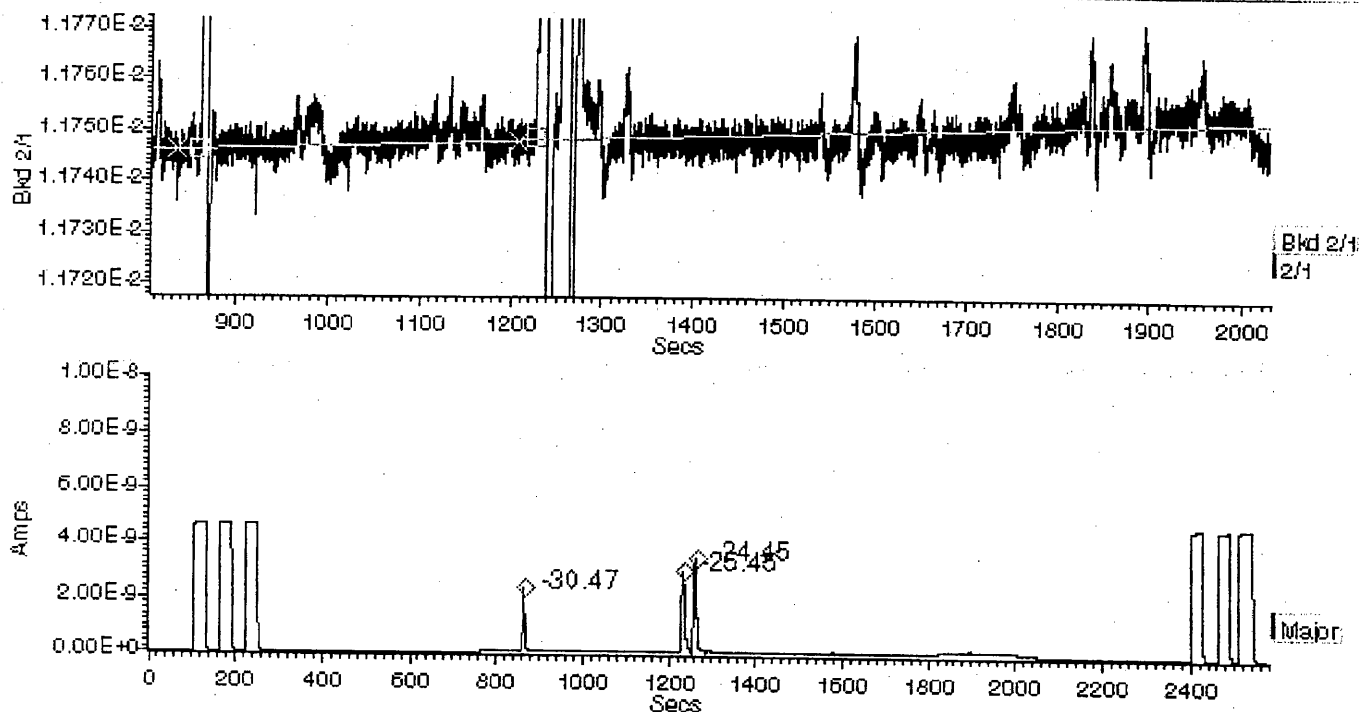
| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 869.0 | 2.38E-9 | 1.1854E-8 | 1.1814E-2 | 4.1671E-3 | -30.47 | -71.32 | -39.06 | 27 |
| 1241.0 | 3.05E-9 | 2.5545E-8 | 1.1871E-2 | 4.1680E-3 | -25.45 | -69.40 | -38.80 | 27 |
| 1267.5 | 3.48E-9 | 2.8518E-8 | 1.1882E-2 | 4.1675E-3 | -24.45 | -69.24 | -38.93 | 27 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Filename : DATA_014 Folder : 040806
 Date : 04/08/06 Time : 20:02:53
 Comment : Blu 1 Pool 4 F2/1400uL inj 2uL
 Parameters Automatic DP Params

√ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

119

Data Processing Results

Data File Name : DATA_014
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F2/1400uL inj 2uL
 Sample ID :
 Sample Position : 7
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 20:02:53 Date : 04/08/06
 Current Time : 10:45:06 Date : 05/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.496E-8 | 1.1776E-2 | 4.2531E-3 |
| 182.6 | 8.503E-8 | 1.1776E-2 | 4.2531E-3 |
| 242.6 | 8.494E-8 | 1.1776E-2 | 4.2528E-3 |
| 2423.5 | 8.442E-8 | 1.1774E-2 | 4.2519E-3 |
| 2483.5 | 8.385E-8 | 1.1774E-2 | 4.2515E-3 |
| 2533.5 | 8.462E-8 | 1.1773E-2 | 4.2518E-3 |

Std Dev Of Fit 3.6403E-7 1.7354E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 869.0 | 2.38E-9 | 1.2861E-8 | 1.1814E-2 | 4.1675E-3 | -30.47 | -71.51 | -38.95 | 27 |
| | 1241.0 | 3.05E-9 | 2.5235E-8 | 1.1869E-2 | 4.1679E-3 | -25.56 | -68.92 | -38.82 | 27 |
| | 1267.5 | 3.48E-9 | 2.8807E-8 | 1.1881E-2 | 4.1677E-3 | -24.53 | -68.67 | -38.88 | 27 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

GDC00989

129

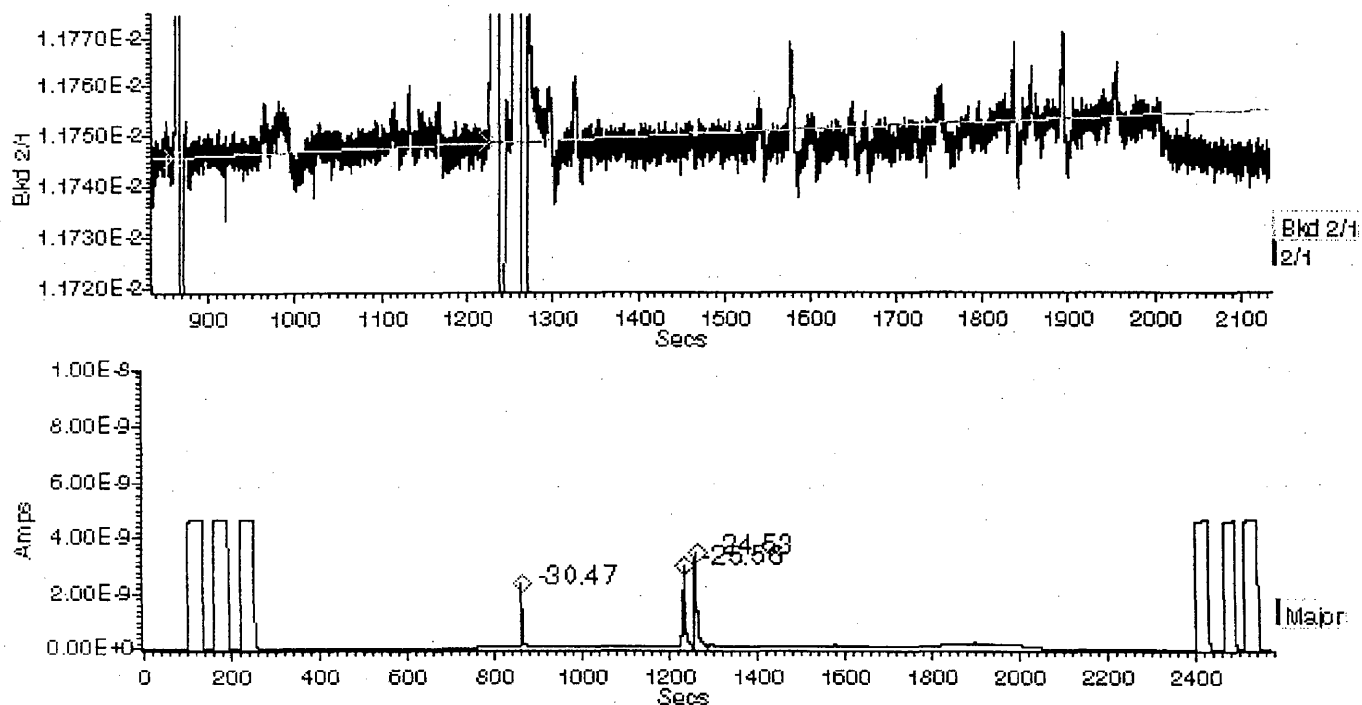
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_014 Folder : 040806
 Date : 04/08/06 Time : 20:02:53
 Comment : Blu 1 Pool 4 F2/1400uL inj 2uL
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

121

Data Processing Results

Data File Name : DATA_014
 Folder : 040806
 Sample Name : Blu 1 Pool 4 F2/1400uL inj 2uL
 Sample ID :
 Sample Position : 7
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 20:02:53 Date : 04/08/06
 Current Time : 10:43:29 Date : 05/05/07

Zero

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.496E-8 | 1.1776E-2 | 4.2531E-3 |
| 182.6 | 8.503E-8 | 1.1776E-2 | 4.2531E-3 |
| 242.6 | 8.494E-8 | 1.1776E-2 | 4.2528E-3 |
| 2423.5 | 8.442E-8 | 1.1774E-2 | 4.2519E-3 |
| 2483.5 | 8.385E-8 | 1.1774E-2 | 4.2515E-3 |
| 2533.5 | 8.462E-8 | 1.1773E-2 | 4.2518E-3 |

Std Dev Of Fit 3.6403E-7 1.7354E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 869.0 | 2.38E-9 | 1.2861E-8 | 1.1818E-2 | 4.1700E-3 | -30.14 | -38.38 > |
| 1241.0 | 3.05E-9 | 2.5235E-8 | 1.1870E-2 | 4.1668E-3 | -25.49 | -39.09<> |
| 1267.5 | 3.48E-9 | 2.8807E-8 | 1.1881E-2 | 4.1662E-3 | -24.51 | -39.23< |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

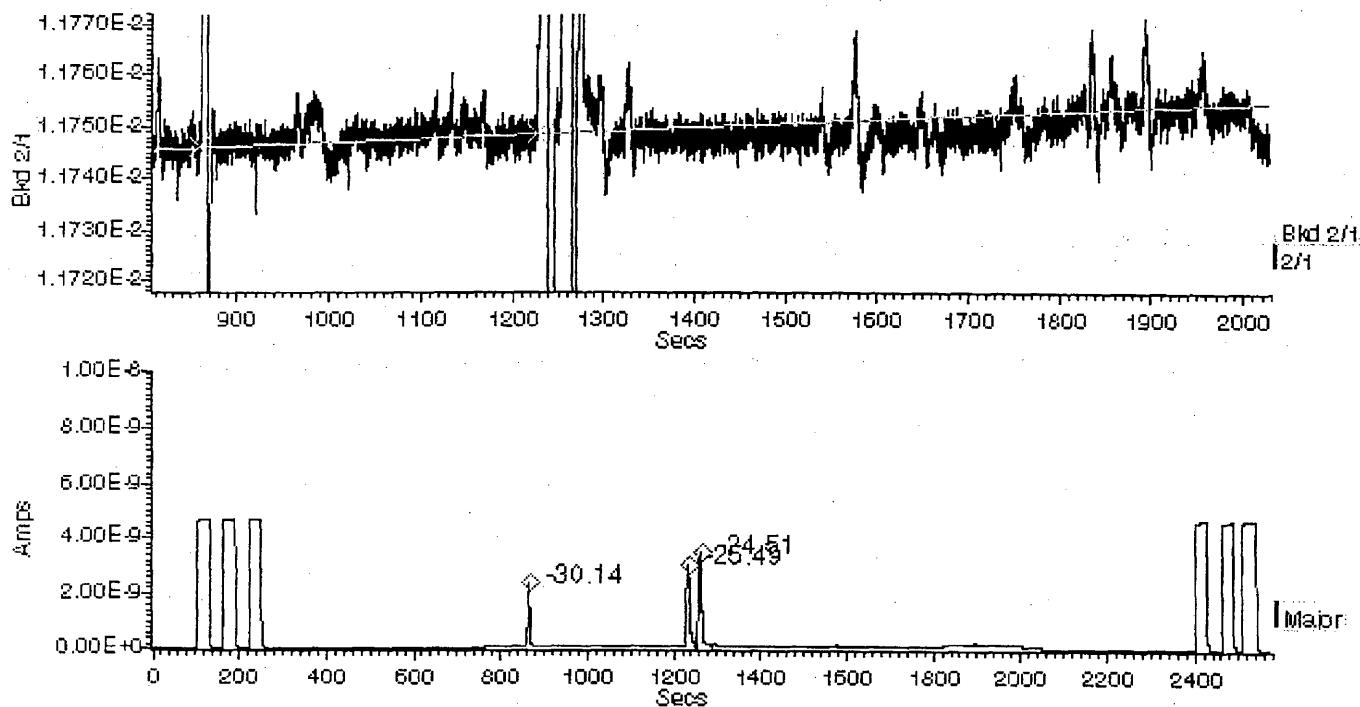
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_014 Folder : 040806
 Date : 04/08/06 Time : 20:02:53
 Comment : Blu 1 Pool 4 F2/1400uL inj 2uL
 Parameters Automatic DP Params

▾ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

123

Data Processing Results

Data File Name : DATA_015
 Folder : 040806
 Sample Name : 178/07 995474 F2/850uL inj 2uL
 Sample ID :
 Sample Position : 8
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 20:47:38 Date : 04/08/06
 Current Time : 10:50:40 Date : 05/05/07

Manual.

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.556E-8 | 1.1774E-2 | 4.2526E-3 |
| 182.5 | 8.604E-8 | 1.1775E-2 | 4.2533E-3 |
| 242.5 | 8.602E-8 | 1.1775E-2 | 4.2537E-3 |
| 2423.4 | 8.506E-8 | 1.1773E-2 | 4.2517E-3 |
| 2483.5 | 8.522E-8 | 1.1773E-2 | 4.2517E-3 |
| 2533.5 | 8.602E-8 | 1.1773E-2 | 4.2523E-3 |

Std Dev Of Fit 5.2841E-7 4.9662E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 870.1 | 3.47E-9 | 1.7634E-8 | 1.1817E-2 | 4.1656E-3 | -30.07 | -64.46 | -39.43 | 21 |
| 1241.1 | 4.00E-9 | 3.2222E-8 | 1.1858E-2 | 4.1655E-3 | -26.46 | -62.25 | -39.41 | 21 |
| 1266.9 | 2.92E-9 | 2.2808E-8 | 1.1845E-2 | 4.1691E-3 | -27.60 | -62.03 | -38.57 | 21 |
| 1301.9 | 3.84E-9 | 3.2903E-8 | 1.1796E-2 | 4.1718E-3 | -31.93 | -61.72 | -37.96 | 20 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

DP Optima GC 1.67-2 - Manual DP

□ □

File Edit View Calculate Report Parameters Status Help

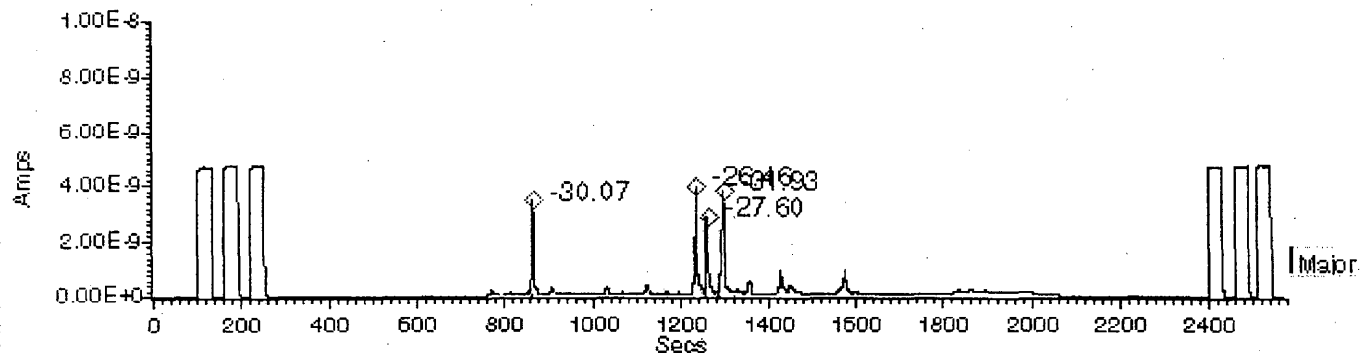
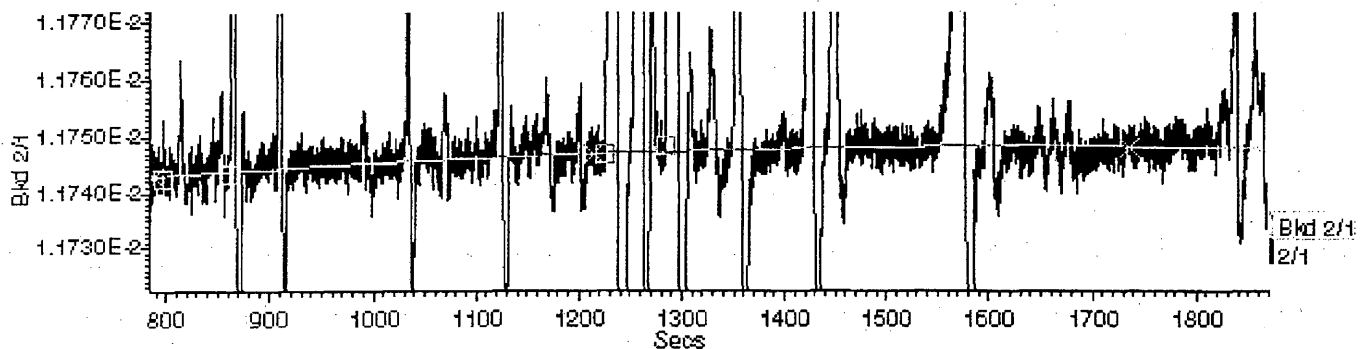
Data Filename : DATA_015 Folder : 040806
 Date : 04/08/06 Time : 20:47:38
 Comment : 178/07 995474 F2/850uL inj 2uL

Parameters Automatic DP Params

× Data Processing Main Graph

□ □

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

125

Data Processing Results

Data File Name : DATA_015
 Folder : 040806
 Sample Name : 178/07 995474 F2/850uL inj 2uL
 Sample ID :
 Sample Position : 8
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 20:47:38 Date : 04/08/06
 Current Time : 10:47:17 Date : 05/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.556E-8 | 1.1774E-2 | 4.2526E-3 |
| 182.5 | 8.604E-8 | 1.1775E-2 | 4.2533E-3 |
| 242.5 | 8.602E-8 | 1.1775E-2 | 4.2537E-3 |
| 2423.4 | 8.506E-8 | 1.1773E-2 | 4.2517E-3 |
| 2483.5 | 8.522E-8 | 1.1773E-2 | 4.2517E-3 |
| 2533.5 | 8.602E-8 | 1.1773E-2 | 4.2523E-3 |

Std Dev Of Fit 5.2841E-7 4.9662E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 870.1 | 3.47E-9 | 1.7634E-8 | 1.1818E-2 | 4.1656E-3 | -29.94 | -65.01 | -39.43 | 21 |
| 1241.1 | 4.00E-9 | 3.2222E-8 | 1.1858E-2 | 4.1655E-3 | -26.47 | -62.15 | -39.41 | 21 |
| 1266.9 | 2.92E-9 | 2.2808E-8 | 1.1844E-2 | 4.1691E-3 | -27.69 | -61.61 | -38.57 | 21 |
| 1301.9 | 3.84E-9 | 3.2903E-8 | 1.1794E-2 | 4.1718E-3 | -32.08 | -60.85 | -37.96 | 20 |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

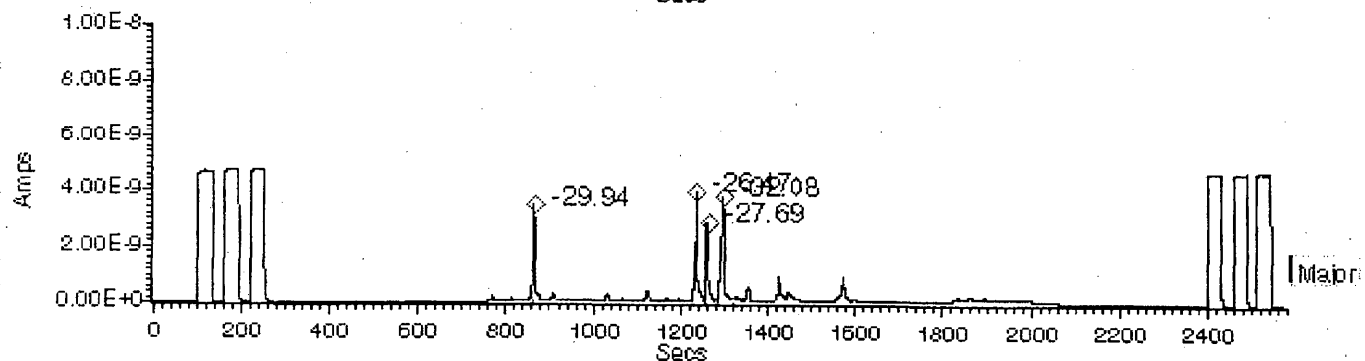
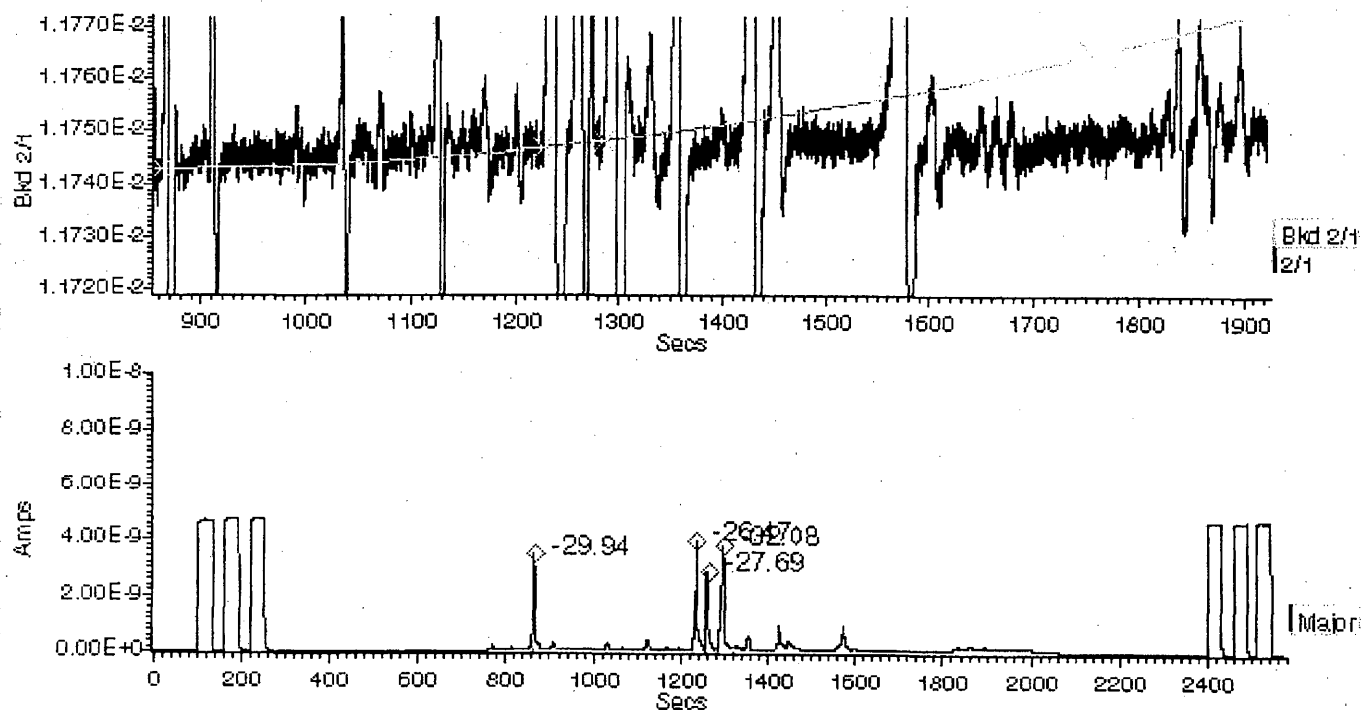
File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_015 Folder : 040806
 Date : 04/08/06 Time : 20:47:38
 Comment : 178/07 995474 F2/850uL inj 2uL
 Parameters Automatic DP Params

Data Processing Main Graph

101

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

127

Data Processing Results

Data File Name : DATA 015
 Folder : 040806
 Sample Name : 178/07 995474 F2/850uL inj 2uL
 Sample ID :
 Sample Position : 8
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 20:47:38 Date : 04/08/06
 Current Time : 10:51:50 Date : 05/05/07

Zero

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.6 | 8.556E-8 | 1.1774E-2 | 4.2526E-3 |
| 182.5 | 8.604E-8 | 1.1775E-2 | 4.2533E-3 |
| 242.5 | 8.602E-8 | 1.1775E-2 | 4.2537E-3 |
| 2423.4 | 8.506E-8 | 1.1773E-2 | 4.2517E-3 |
| 2483.5 | 8.522E-8 | 1.1773E-2 | 4.2517E-3 |
| 2533.5 | 8.602E-8 | 1.1773E-2 | 4.2523E-3 |

Std Dev Of Fit 5.2841E-7 4.9662E-7

Analysis of Sample Peaks, with Zero Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|-----|--------|---------|-----------|-----------|-----------|--------|----------|
| | 870.1 | 3.47E-9 | 1.7634E-8 | 1.1815E-2 | 4.1649E-3 | -30.18 | -39.61 > |
| | 1241.1 | 4.00E-9 | 3.2222E-8 | 1.1856E-2 | 4.1655E-3 | -26.58 | -39.42<> |
| | 1266.9 | 2.92E-9 | 2.2808E-8 | 1.1845E-2 | 4.1664E-3 | -27.62 | -39.22<> |
| | 1301.9 | 3.84E-9 | 3.2903E-8 | 1.1801E-2 | 4.1660E-3 | -31.42 | -39.29< |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

GDC00997

128

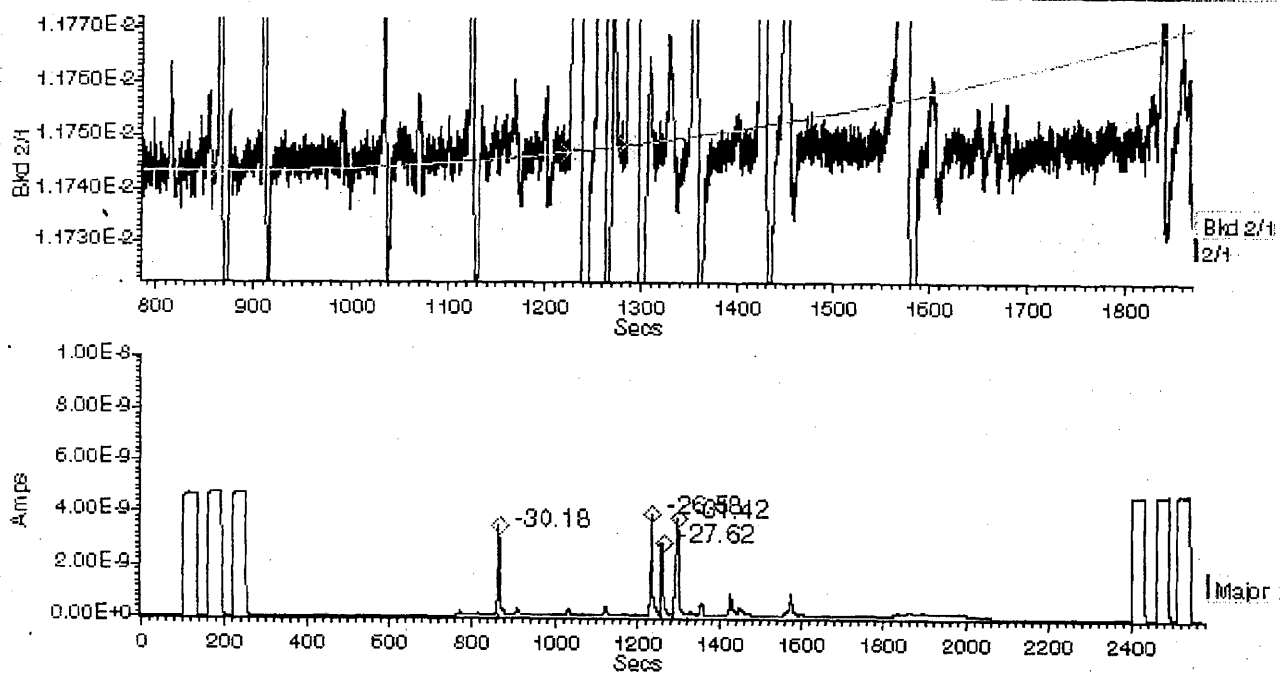
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_015 Folder : 040806
 Date : 04/08/06 Time : 20:47:38
 Comment : 178107 995474 F2/850uL inj 2uL
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

GDC00998

129

Data Processing Results

Data File Name : DATA_016
 Folder : 040806
 Sample Name : Mix Cal Acetate 001A-100ng inj
 Sample ID :
 Sample Position : 9
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 21:32:21 Date : 04/08/06
 Current Time : 10:59:41 Date : 05/05/07

Manual.

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.534E-8 | 1.1775E-2 | 4.2534E-3 |
| 182.6 | 8.513E-8 | 1.1775E-2 | 4.2533E-3 |
| 242.6 | 8.502E-8 | 1.1774E-2 | 4.2532E-3 |
| 2423.4 | 8.433E-8 | 1.1774E-2 | 4.2522E-3 |
| 2483.4 | 8.404E-8 | 1.1774E-2 | 4.2518E-3 |
| 2533.5 | 8.484E-8 | 1.1773E-2 | 4.2521E-3 |

Std Dev Of Fit 4.6118E-7 1.1639E-7

Analysis of Sample Peaks, with Background Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| 870.5 | 4.31E-9 | 1.9411E-8 | 1.1813E-2 | 4.1647E-3 | -30.42 | -77.27 | -39.67 | 34 |
| 1241.6 | 4.22E-9 | 3.4762E-8 | 1.1931E-2 | 4.1678E-3 | -20.07 | -76.12 | -38.92 | 33 |
| 1316.1 | 3.28E-9 | 2.6681E-8 | 1.1775E-2 | 4.1689E-3 | -33.77 | -76.10 | -38.64 | 32 |
| 1490.4 | 2.97E-9 | 3.0830E-8 | 1.1968E-2 | 4.1691E-3 | -16.88 | -76.24 | -38.60 | 32 |

COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

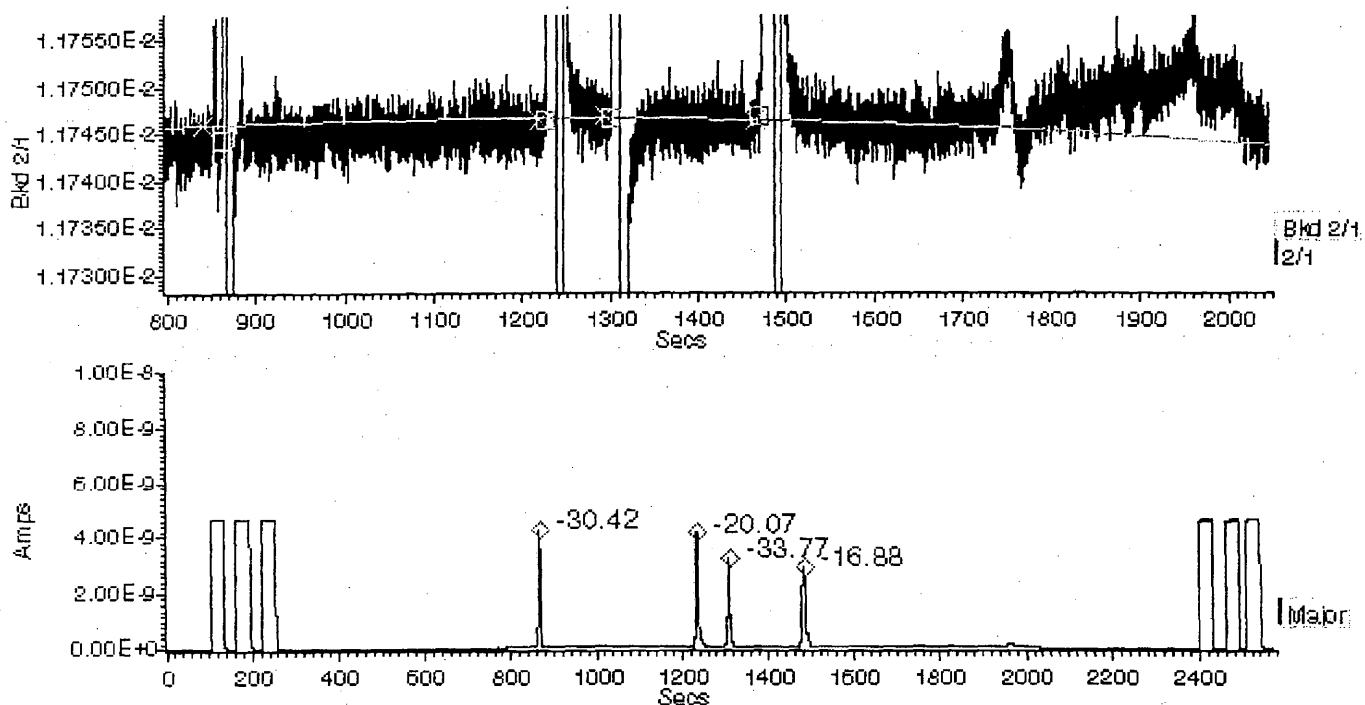
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename: DATA_016 Folder: 040806
 Date: 04/08/06 Time: 21:32:21
 Comment: Mix Cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

x Data Processing Main Graph

Graph Cursor Lines Window



CONFIDENTIAL
 FORMULAIRES ORIGINALE

131

Data Processing Results

Data File Name : DATA_016
 Folder : 040806
 Sample Name : Mix Cal Acetate 001A-100ng inj
 Sample ID :
 Sample Position : 9
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 21:32:21 Date : 04/08/06
 Current Time : 10:53:50 Date : 05/05/07

Auto

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.534E-8 | 1.1775E-2 | 4.2534E-3 |
| 182.6 | 8.513E-8 | 1.1775E-2 | 4.2533E-3 |
| 242.6 | 8.502E-8 | 1.1774E-2 | 4.2532E-3 |
| 2423.4 | 8.433E-8 | 1.1774E-2 | 4.2522E-3 |
| 2483.4 | 8.404E-8 | 1.1774E-2 | 4.2518E-3 |
| 2533.5 | 8.484E-8 | 1.1773E-2 | 4.2521E-3 |

Std Dev Of Fit 4.6118E-7 1.1639E-7

Analysis of Sample Peaks, with Background Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC13Bkd | dO18Pk | dO18 |
|-----|--------|---------|-----------|-----------|-----------|--------|---------|--------|------|
| | 870.5 | 4.31E-9 | 1.9411E-8 | 1.1815E-2 | 4.1647E-3 | -30.26 | -78.95 | -39.67 | 34 |
| | 1241.6 | 4.22E-9 | 3.5964E-8 | 1.1931E-2 | 4.1687E-3 | -20.13 | -76.35 | -38.72 | 32 |
| | 1316.1 | 3.28E-9 | 2.7273E-8 | 1.1775E-2 | 4.1695E-3 | -33.81 | -76.09 | -38.50 | 32 |
| | 1490.4 | 2.97E-9 | 3.2050E-8 | 1.1965E-2 | 4.1704E-3 | -17.13 | -75.64 | -38.31 | 32 |

COPIES
 CONSERVÉES
 ET FORMULAIRES ORIGINAUX

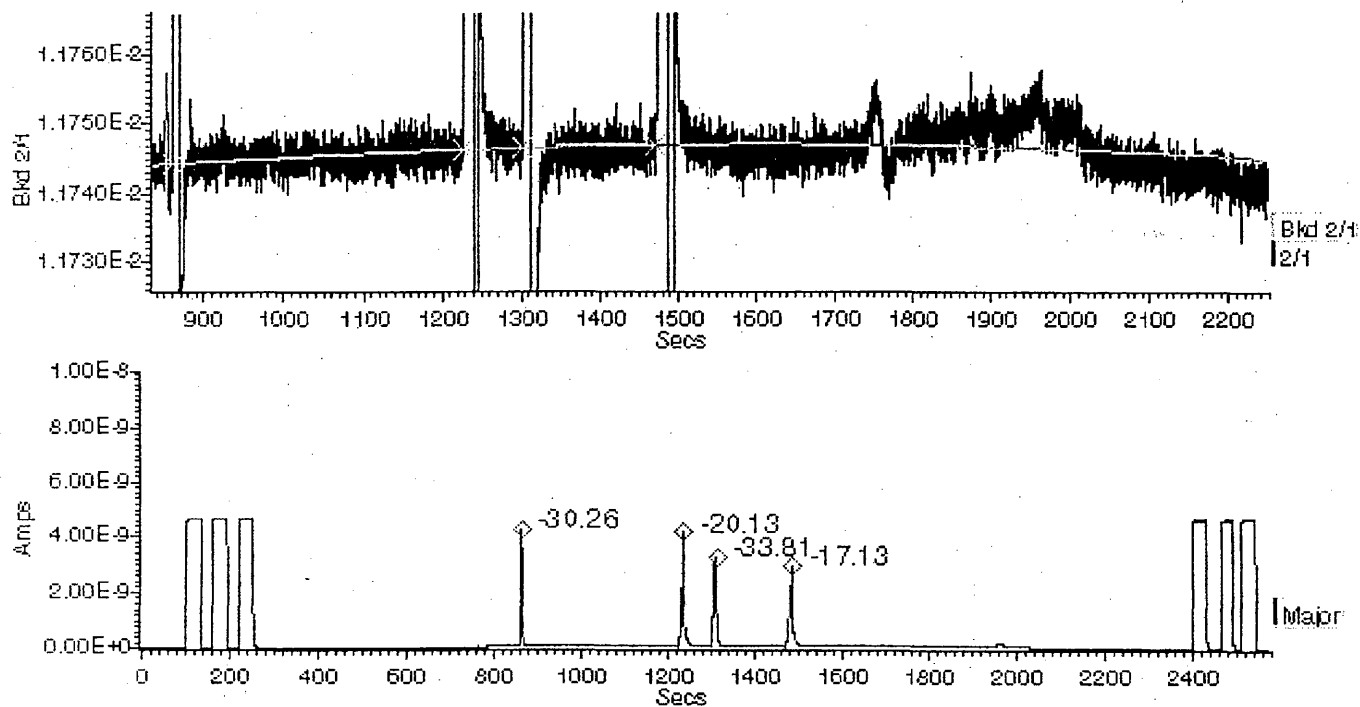
DP Optima GC 1.67-2 - Manual DP

File Edit View Calculate Report Parameters Status Help

Data Filename : DATA_016 Folder : 040806
 Date : 04/08/06 Time : 21:32:21
 Comment : Mix Cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

≡ Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

133

Data Processing Results

Data File Name : DATA 016
 Folder : 040806
 Sample Name : Mix Cal Acetate 001A-100ng inj
 Sample ID :
 Sample Position : 9
 Injection Size : 0.0000
 Sample Type : Sam
 Method : M-AN-41
 Batch Name :
 RunTime User : micromass
 Acquisition Time : 21:32:21 Date : 04/08/06
 Current Time : 11:00:53 Date : 05/05/07

Zero

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|--------|----------|-----------|-----------|
| 122.5 | 8.534E-8 | 1.1775E-2 | 4.2534E-3 |
| 182.6 | 8.513E-8 | 1.1775E-2 | 4.2533E-3 |
| 242.6 | 8.502E-8 | 1.1774E-2 | 4.2532E-3 |
| 2423.4 | 8.433E-8 | 1.1774E-2 | 4.2522E-3 |
| 2483.4 | 8.404E-8 | 1.1774E-2 | 4.2518E-3 |
| 2533.5 | 8.484E-8 | 1.1773E-2 | 4.2521E-3 |

Std Dev Of Fit 4.6118E-7 1.1639E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|--------|---------|-----------|-----------|-----------|--------|----------|
| 870.5 | 4.31E-9 | 1.9411E-8 | 1.1817E-2 | 4.1667E-3 | -30.13 | -39.21 > |
| 1241.6 | 4.22E-9 | 3.5964E-8 | 1.1927E-2 | 4.1654E-3 | -20.38 | -39.49<> |
| 1316.1 | 3.28E-9 | 2.7273E-8 | 1.1784E-2 | 4.1648E-3 | -32.91 | -39.59<> |
| 1490.4 | 2.97E-9 | 3.2050E-8 | 1.1959E-2 | 4.1638E-3 | -17.59 | -39.82< |

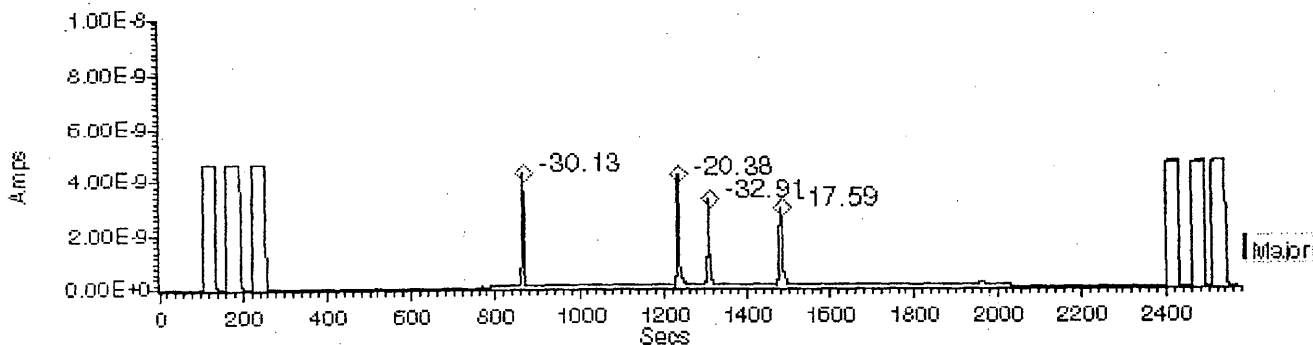
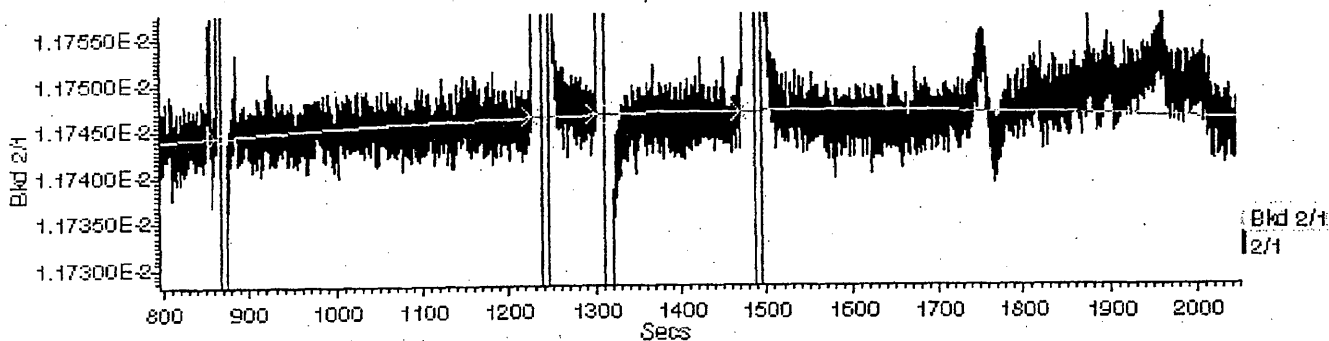
COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

File Edit View Calculate Report Parameters Status Help

Data Filename: DATA_016 Folder: 040806
 Date: 04/08/06 Time: 21:32:21
 Comment: Mix Cal Acetate 001A-100ng inj
 Parameters Automatic DP Params

Data Processing Main Graph

Graph Cursor Lines Window



COPIE CERTIFIÉE
 CONFORME DES DOCUMENTS
 ET FORMULAIRES ORIGINAUX

135

Data Processing Results

Data File Name : DATA_001
 Folder : 230706
 Sample Name : stabilite 1
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-STAB
 Batch Name : 230706
 RunTime User : micromass
 Acquisition Time : 09:28:35 Date : 23/07/06
 Current Time : 11:11:57 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.5 | 8.399E-8 | 1.1783E-2 | 4.2479E-3 |
| 102.5 | 8.397E-8 | 1.1783E-2 | 4.2477E-3 |
| 162.6 | 8.393E-8 | 1.1782E-2 | 4.2476E-3 |
| 222.6 | 8.382E-8 | 1.1782E-2 | 4.2473E-3 |
| 282.6 | 8.388E-8 | 1.1782E-2 | 4.2476E-3 |
| 342.6 | 8.404E-8 | 1.1781E-2 | 4.2474E-3 |
| 402.7 | 8.420E-8 | 1.1781E-2 | 4.2479E-3 |
| 462.7 | 8.536E-8 | 1.1780E-2 | 4.2483E-3 |
| 522.7 | 8.544E-8 | 1.1780E-2 | 4.2485E-3 |
| 582.7 | 8.550E-8 | 1.1780E-2 | 4.2489E-3 |

Std Dev Of Fit 3.0436E-7 3.8588E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Data File Name : DATA_002
 Folder : 230706
 Sample Name : stabilite 2
 Sample ID :
 Sample Position : 2
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-STAB
 Batch Name : 230706
 RunTime User : micromass
 Acquisition Time : 09:39:43 Date : 23/07/06
 Current Time : 11:12:54 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.5 | 8.495E-8 | 1.1781E-2 | 4.2496E-3 |
| 102.5 | 8.535E-8 | 1.1781E-2 | 4.2497E-3 |
| 162.5 | 8.542E-8 | 1.1781E-2 | 4.2500E-3 |
| 222.5 | 8.542E-8 | 1.1781E-2 | 4.2500E-3 |
| 282.5 | 8.492E-8 | 1.1781E-2 | 4.2501E-3 |
| 342.6 | 8.488E-8 | 1.1781E-2 | 4.2500E-3 |
| 402.6 | 8.485E-8 | 1.1781E-2 | 4.2501E-3 |
| 462.6 | 8.465E-8 | 1.1780E-2 | 4.2497E-3 |
| 522.7 | 8.419E-8 | 1.1780E-2 | 4.2495E-3 |
| 582.7 | 8.430E-8 | 1.1780E-2 | 4.2495E-3 |

Std Dev Of Fit 1.7641E-7 2.3112E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME AUX DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Data File Name : DATA_003
 Folder : 230706
 Sample Name : stabilite 3
 Sample ID :
 Sample Position : 3
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-STAB
 Batch Name : 230706
 RunTime User : micromass
 Acquisition Time : 09:50:48 Date : 23/07/06
 Current Time : 11:13:10 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.5 | 8.459E-8 | 1.1780E-2 | 4.2498E-3 |
| 102.5 | 8.466E-8 | 1.1780E-2 | 4.2496E-3 |
| 162.5 | 8.456E-8 | 1.1780E-2 | 4.2494E-3 |
| 222.6 | 8.442E-8 | 1.1780E-2 | 4.2494E-3 |
| 282.6 | 8.499E-8 | 1.1779E-2 | 4.2495E-3 |
| 342.6 | 8.562E-8 | 1.1779E-2 | 4.2498E-3 |
| 402.6 | 8.627E-8 | 1.1779E-2 | 4.2500E-3 |
| 462.7 | 8.632E-8 | 1.1780E-2 | 4.2503E-3 |
| 522.7 | 8.640E-8 | 1.1780E-2 | 4.2506E-3 |
| 582.7 | 8.618E-8 | 1.1780E-2 | 4.2511E-3 |

Std Dev Of Fit 5.2114E-7 3.2526E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

138

Data Processing Results

Data File Name : DATA_001
 Folder : 040806
 Sample Name : Stabilite 1
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-STAB
 Batch Name : 040806
 RunTime User : micromass
 Acquisition Time : 10:24:23 Date : 04/08/06
 Current Time : 11:14:05 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.5 | 8.364E-8 | 1.1784E-2 | 4.2523E-3 |
| 102.5 | 8.361E-8 | 1.1784E-2 | 4.2519E-3 |
| 162.6 | 8.334E-8 | 1.1783E-2 | 4.2516E-3 |
| 222.6 | 8.367E-8 | 1.1783E-2 | 4.2513E-3 |
| 282.6 | 8.395E-8 | 1.1782E-2 | 4.2515E-3 |
| 342.6 | 8.457E-8 | 1.1781E-2 | 4.2514E-3 |
| 402.6 | 8.458E-8 | 1.1781E-2 | 4.2516E-3 |
| 462.7 | 8.502E-8 | 1.1781E-2 | 4.2519E-3 |
| 522.7 | 8.476E-8 | 1.1781E-2 | 4.2518E-3 |
| 582.7 | 8.461E-8 | 1.1781E-2 | 4.2520E-3 |

Std Dev Of Fit 4.0313E-7 3.1520E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Data File Name : DATA_002
 Folder : 040806
 Sample Name : Stabilite 2
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-STAB
 Batch Name : 040806
 RunTime User : micromass
 Acquisition Time : 10:35:30 Date : 04/08/06
 Current Time : 11:14:21 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|------------|-----------|
| 42.5 | 8.480E-8 | 1.1782E-2 | 4.2530E-3 |
| 102.5 | 8.483E-8 | 1.1782E-2 | 4.2531E-3 |
| 162.5 | 8.466E-8 | 1.1782E-2 | 4.2532E-3 |
| 222.5 | 8.449E-8 | 1.1782E-2* | 4.2531E-3 |
| 282.6 | 8.459E-8 | 1.1781E-2 | 4.2531E-3 |
| 342.6 | 8.429E-8 | 1.1781E-2 | 4.2529E-3 |
| 402.6 | 8.397E-8 | 1.1781E-2 | 4.2527E-3 |
| 462.7 | 8.360E-8 | 1.1781E-2 | 4.2526E-3 |
| 522.7 | 8.368E-8 | 1.1780E-2 | 4.2524E-3 |
| 582.7 | 8.357E-8 | 1.1780E-2 | 4.2520E-3 |

Std Dev Of Fit

1.6708E-7

2.0929E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dC18Pk |
|-------|--------|------|-----|-----|--------|--------|
| ----- | | | | | | |

COPIE CERTIFIEE
 CONFORME AUX LOIS
 ET FORMULAIRES ORIGINAUX

GDC01009

Data Processing Results

Data File Name : DATA_003
 Folder : 040806
 Sample Name : Stabilite 3
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-STAB
 Batch Name : 040806
 RunTime User : micromass
 Acquisition Time : 10:46:35 Date : 04/08/06
 Current Time : 11:15:00 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.5 | 8.385E-8 | 1.1780E-2 | 4.2523E-3 |
| 102.5 | 8.449E-8 | 1.1779E-2 | 4.2523E-3 |
| 162.5 | 8.486E-8 | 1.1779E-2 | 4.2523E-3 |
| 222.5 | 8.492E-8 | 1.1779E-2 | 4.2526E-3 |
| 282.6 | 8.492E-8 | 1.1779E-2 | 4.2528E-3 |
| 342.6 | 8.472E-8 | 1.1779E-2 | 4.2530E-3 |
| 402.6 | 8.471E-8 | 1.1780E-2 | 4.2532E-3 |
| 462.6 | 8.487E-8 | 1.1780E-2 | 4.2536E-3 |
| 522.6 | 8.495E-8 | 1.1780E-2 | 4.2541E-3 |
| 582.7 | 8.515E-8 | 1.1781E-2 | 4.2544E-3 |

Std Dev Of Fit 4.7259E-7 2.0677E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | d018Pk |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Data File Name : DATA_004
 Folder : 040806
 Sample Name : Stabilite 4
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-STAB
 Batch Name : 040806
 RunTime User : micromass
 Acquisition Time : 10:57:40 Date : 04/08/06
 Current Time : 11:15:20 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.5 | 8.426E-8 | 1.1781E-2 | 4.2541E-3 |
| 102.5 | 8.413E-8 | 1.1781E-2 | 4.2541E-3 |
| 162.5 | 8.397E-8 | 1.1781E-2 | 4.2538E-3 |
| 222.6 | 8.358E-8 | 1.1781E-2 | 4.2537E-3 |
| 282.6 | 8.378E-8 | 1.1781E-2 | 4.2534E-3 |
| 342.7 | 8.406E-8 | 1.1780E-2 | 4.2530E-3 |
| 402.6 | 8.377E-8 | 1.1780E-2 | 4.2527E-3 |
| 462.7 | 8.380E-8 | 1.1779E-2 | 4.2527E-3 |
| 522.7 | 8.407E-8 | 1.1779E-2 | 4.2526E-3 |
| 582.7 | 8.494E-8 | 1.1778E-2 | 4.2527E-3 |

Std Dev Of Fit 2.6648E-7 1.8416E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | 2013PK | 2018PK |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Data File Name : DATA_005
 Folder : 040806
 Sample Name : Stabilite 5
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-STAB
 Batch Name : 040806
 RunTime User : micromass
 Acquisition Time : 11:08:45 Date : 04/08/06
 Current Time : 11:15:30 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.5 | 8.495E-8 | 1.1779E-2 | 4.2534E-3 |
| 102.5 | 8.499E-8 | 1.1779E-2 | 4.2535E-3 |
| 162.6 | 8.506E-8 | 1.1779E-2 | 4.2539E-3 |
| 222.6 | 8.502E-8 | 1.1779E-2 | 4.2538E-3 |
| 282.6 | 8.494E-8 | 1.1779E-2 | 4.2542E-3 |
| 342.6 | 8.489E-8 | 1.1779E-2 | 4.2543E-3 |
| 402.6 | 8.451E-8 | 1.1779E-2 | 4.2542E-3 |
| 462.7 | 8.487E-8 | 1.1779E-2 | 4.2543E-3 |
| 522.7 | 8.458E-8 | 1.1779E-2 | 4.2540E-3 |
| 582.7 | 8.439E-8 | 1.1779E-2 | 4.2538E-3 |

Std Dev Of Fit 1.7272E-7 2.7766E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

GDC01012

Data Processing Results

Data File Name : DATA_008
 Folder : 260606
 Sample Name : linearite 1
 Sample ID :
 Sample Position : 8
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-LIN
 Batch Name : 260606
 RunTime User : micromass
 Acquisition Time : 11:23:11 Date : 26/06/06
 Current Time : 11:15:42 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.5 | 2.876E-8 | 1.1790E-2 | 4.2592E-3 |
| 102.5 | 2.875E-8 | 1.1789E-2 | 4.2590E-3 |
| 162.5 | 1.082E-7 | 1.1792E-2 | 4.2579E-3 |
| 222.6 | 1.077E-7 | 1.1792E-2 | 4.2579E-3 |
| 282.6 | 7.264E-8 | 1.1791E-2 | 4.2585E-3 |
| 342.6 | 7.256E-8 | 1.1791E-2 | 4.2581E-3 |
| 402.6 | 1.704E-7 | 1.1792E-2 | 4.2571E-3 |
| 462.6 | 1.702E-7 | 1.1792E-2 | 4.2573E-3 |
| 522.7 | 2.668E-8 | 1.1788E-2 | 4.2588E-3 |
| 582.7 | 2.692E-8 | 1.1788E-2 | 4.2586E-3 |

2.3 nA
1.5 nA

Std Dev Of Fit 1.6725E-6 6.9427E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES CHIFFRES
 ET SÉRIELS ORIGINAUX

Data Processing Results

Data File Name : DATA_009
 Folder : 260606
 Sample Name : linearite 2
 Sample ID :
 Sample Position : 9
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-LIN
 Batch Name : 260606
 RunTime User : micromass
 Acquisition Time : 11:35:39 Date : 26/06/06
 Current Time : 11:15:54 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.6 | 2.687E-8 | 1.1788E-2 | 4.2591E-3 |
| 102.6 | 2.689E-8 | 1.1788E-2 | 4.2588E-3 |
| 162.6 | 1.089E-7 | 1.1792E-2 | 4.2581E-3 |
| 222.6 | 1.085E-7 | 1.1791E-2 | 4.2583E-3 |
| 282.6 | 6.987E-8 | 1.1791E-2 | 4.2588E-3 |
| 342.6 | 6.988E-8 | 1.1791E-2 | 4.2588E-3 |
| 402.7 | 1.763E-7 | 1.1792E-2 | 4.2572E-3 |
| 462.7 | 1.766E-7 | 1.1792E-2 | 4.2574E-3 |
| 522.7 | 2.703E-8 | 1.1788E-2 | 4.2594E-3 |
| 582.8 | 2.709E-8 | 1.1788E-2 | 4.2591E-3 |

} 5.8 nA
 } 1.5 nA

Std Dev Of Fit 1.8697E-6 7.7341E-7

Analysis of Sample Peaks, with Zero Subtraction

CO2

| Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|------|--------|------|-----|-----|--------|--------|
|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Data File Name : DATA_010
 Folder : 260606
 Sample Name : linearite 3
 Sample ID :
 Sample Position : 10
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-LIN
 Batch Name : 260606
 RunTime User : micromass
 Acquisition Time : 11:47:05 Date : 26/06/06
 Current Time : 11:16:30 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|--------------------|
| 42.5 | 2.718E-8 | 1.1788E-2 | 4.2594E-3] 1.5 nA |
| 102.5 | 2.719E-8 | 1.1788E-2 | 4.2594E-3] |
| 162.5 | 1.074E-7 | 1.1791E-2 | 4.2579E-3 |
| 222.5 | 1.071E-7 | 1.1790E-2 | 4.2574E-3 |
| 282.6 | 6.932E-8 | 1.1789E-2 | 4.2568E-3 |
| 342.6 | 6.938E-8 | 1.1788E-2 | 4.2569E-3 |
| 402.6 | 1.790E-7 | 1.1790E-2 | 4.2559E-3] 9.9 nA |
| 462.6 | 1.791E-7 | 1.1790E-2 | 4.2561E-3] |
| 522.7 | 2.856E-8 | 1.1787E-2 | 4.2571E-3 |
| 582.7 | 2.863E-8 | 1.1787E-2 | 4.2567E-3 |

Std Dev Of Fit 1.3543E-6 7.4675E-7

Analysis of Sample Peaks, with Zero Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|-------|-------|--------|-------|-------|-------|--------|--------|
| ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Data File Name : DATA_007
 Folder : 310706
 Sample Name : Linearite 1
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-LIN
 Batch Name : 310706
 RunTime User : micromass
 Acquisition Time : 11:58:36 Date : 31/07/06
 Current Time : 11:20:13 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.6 | 3.050E-8 | 1.1777E-2 | 4.2512E-3 |
| 102.6 | 3.050E-8 | 1.1777E-2 | 4.2511E-3 |
| 162.6 | 1.235E-7 | 1.1779E-2 | 4.2496E-3 |
| 222.6 | 1.230E-7 | 1.1778E-2 | 4.2498E-3 |
| 282.7 | 7.399E-8 | 1.1779E-2 | 4.2507E-3 |
| 342.7 | 7.403E-8 | 1.1779E-2 | 4.2508E-3 |
| 402.7 | 1.783E-7 | 1.1778E-2 | 4.2491E-3 |
| 462.7 | 1.775E-7 | 1.1779E-2 | 4.2493E-3 |
| 522.8 | 2.966E-8 | 1.1777E-2 | 4.2504E-3 |
| 582.8 | 2.978E-8 | 1.1776E-2 | 4.2505E-3 |

9.8 nA
1.6 nA

Std Dev Of Fit 1.1321E-6 7.4206E-7

Analysis of Sample Peaks, with Zero Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|-----|------|--------|------|-----|-----|--------|--------|
|-----|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

147

Data Processing Results

Data File Name : DATA_008
 Folder : 310706
 Sample Name : Linearite 2
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-LIN
 Batch Name : 310706
 RunTime User : micromass
 Acquisition Time : 12:10:02 Date : 31/07/06
 Current Time : 11:21:03 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.6 | 3.001E-8 | 1.1777E-2 | 4.2512E-3 |
| 102.6 | 3.008E-8 | 1.1776E-2 | 4.2513E-3 |
| 162.6 | 1.317E-7 | 1.1779E-2 | 4.2502E-3 |
| 222.6 | 1.321E-7 | 1.1779E-2 | 4.2502E-3 |
| 282.6 | 7.124E-8 | 1.1778E-2 | 4.2515E-3 |
| 342.6 | 7.105E-8 | 1.1778E-2 | 4.2510E-3 |
| 402.7 | 1.844E-7 | 1.1778E-2 | 4.2492E-3 |
| 462.7 | 1.847E-7 | 1.1778E-2 | 4.2496E-3 |
| 522.7 | 2.984E-8 | 1.1776E-2 | 4.2522E-3 |
| 582.8 | 3.003E-8 | 1.1776E-2 | 4.2514E-3 |

10 nA
1.6 nA

Std Dev Of Fit

1.1051E-6

9.9158E-7

Analysis of Sample Peaks, with Zero Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | d018Pk |
|-----|------|--------|------|-----|-----|--------|--------|
|-----|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

Data Processing Results

Data File Name : DATA_009
 Folder : 310706
 Sample Name : Linearite 3
 Sample ID :
 Sample Position : 1
 Injection Size : 0.0000
 Sample Type : Sam
 Method : CO2-LIN
 Batch Name : 310706
 RunTime User : micromass
 Acquisition Time : 12:21:28 Date : 31/07/06
 Current Time : 11:21:45 Date : 05/05/07

Analysis of Reference Gas Data

Ref Delta 13 = -34.50 Ref Delta 18 = -19.30

| Time | Major | Ratio 2/1 | Ratio 3/1 |
|-------|----------|-----------|-----------|
| 42.6 | 2.990E-8 | 1.1775E-2 | 4.2506E-3 |
| 102.6 | 2.990E-8 | 1.1775E-2 | 4.2503E-3 |
| 162.6 | 1.350E-7 | 1.1777E-2 | 4.2498E-3 |
| 222.6 | 1.358E-7 | 1.1777E-2 | 4.2500E-3 |
| 282.7 | 6.976E-8 | 1.1777E-2 | 4.2511E-3 |
| 342.7 | 6.966E-8 | 1.1777E-2 | 4.2513E-3 |
| 402.7 | 1.818E-7 | 1.1777E-2 | 4.2496E-3 |
| 462.7 | 1.825E-7 | 1.1777E-2 | 4.2496E-3 |
| 522.8 | 2.940E-8 | 1.1776E-2 | 4.2525E-3 |
| 582.8 | 2.955E-8 | 1.1775E-2 | 4.2521E-3 |

10 nA
1.6 nA

Std Dev Of Fit 1.0203E-6 9.6752E-7

Analysis of Sample Peaks, with Zero Subtraction

| CO2 | Time | Height | Area | 2/1 | 3/1 | dC13Pk | dO18Pk |
|-----|------|--------|------|-----|-----|--------|--------|
|-----|------|--------|------|-----|-----|--------|--------|

COPIE CERTIFIEE
 CONFORME DES DONNEES
 ET FORMULAIRES ORIGINAUX

149

Stable Isotope CF Analysis Results

File: DATA_007.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 23-7-2006 10:53:36
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Molecular delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.71 | 122.6 | 1.1781E-02 | 4.2520E-03 |
| 2 | 4.72 | 182.6 | 1.1781E-02 | 4.2521E-03 |
| 3 | 4.72 | 242.7 | 1.1781E-02 | 4.2522E-03 |
| 8 | 4.68 | 2423.5 | 1.1781E-02 | 4.2522E-03 |
| 9 | 4.65 | 2483.6 | 1.1781E-02 | 4.2519E-03 |
| 10 | 4.67 | 2533.5 | 1.1781E-02 | 4.2522E-03 |

Mean: 1.1781E-02 4.2521E-03
Std Dev of fit (%): 0.01 0.04

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 866.9 | 4.68 | 1.8814E-08 | 1.1824E-02 | 3.68 | -30.02 | 4.1647E-03 | -20.55 | -39.48 | -9.84 |
| 2 | 1229.7 | 4.66 | 3.3554E-08 | 1.1942E-02 | 13.71 | -19.67 | 4.1654E-03 | -20.39 | -39.34 | -9.70 |
| 3 | 1302.7 | 3.51 | 2.4451E-08 | 1.1785E-02 | 0.33 | -33.47 | 4.1646E-03 | -20.58 | -39.60 | -9.86 |
| 4 | 1474.1 | 3.06 | 2.8029E-08 | 1.1981E-02 | 17.02 | -16.25 | 4.1652E-03 | -20.43 | -39.39 | -9.75 |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

| | |
|--------------------------------|--------------------------------------|
| File: DATA_008.raw | Acquisition Date: 23-7-2006 11:40:11 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1604.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Co2 stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run index: |
| Description: test de stabilite | |

| Reference standard | Corrections |
|-----------------------------------|-------------------------------|
| Species: CO2 by CF (uncalibrated) | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | |
| Ratio type: Elemental | |
| Deconvolution: Craig | |
| Elemental delta | Molecular delta |
| Label: Value: | Label: Value: wrt: |
| Ratio 1: 13C -34.5 | delta 45 -32.93 PDB |
| Ratio 2: 18O -19.3 | delta 46 -19.35 PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.72 | 122.6 | 1.1780E-02 | 4.2523E-03 |
| 2 | 4.72 | 182.6 | 1.1781E-02 | 4.2525E-03 |
| 3 | 4.72 | 242.6 | 1.1781E-02 | 4.2529E-03 |
| 19 | 4.69 | 2423.6 | 1.1783E-02 | 4.2538E-03 |
| 20 | 4.68 | 2463.5 | 1.1782E-02 | 4.2534E-03 |
| 21 | 4.71 | 2533.5 | 1.1782E-02 | 4.2538E-03 |

Mean: 1.1782E-02 4.2531E-03
Std Dev of fit (%): 0.05 0.06

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 788.2 | 0.96 | 4.7096E-09 | 1.1935E-02 | 12.97 | -20.46 | 4.1699E-03 | -19.56 | -38.54 | -8.86 |
| 2 | 797.3 | 19.72 | 1.1594E-07 | 1.1619E-02 | -13.81 | -50.34 | 4.4557E-03 | 47.64 | 27.49 | 59.20 |
| 3 | 827.5 | 0.94 | 3.0002E-09 | 1.1802E-02 | 1.69 | -32.08 | 4.1662E-03 | -20.42 | -39.35 | -9.71 |
| 4 | 867.3 | 5.92 | 2.3297E-08 | 1.1796E-02 | 1.41 | -32.35 | 4.1644E-03 | -20.86 | -39.78 | -10.15 |
| 5 | 875.3 | 1.01 | 6.4624E-09 | 1.1887E-02 | 8.94 | -24.58 | 4.1656E-03 | -20.58 | -39.52 | -8.88 |
| 6 | 898.1 | 0.41 | 3.1886E-09 | 1.1869E-02 | 7.42 | -26.17 | 4.1676E-03 | -20.10 | -39.05 | -9.40 |
| 7 | 1042.1 | 0.44 | 1.8833E-09 | 1.2030E-02 | 21.09 | -12.11 | 4.1743E-03 | -18.53 | -37.53 | -7.83 |
| 8 | 1045.4 | 0.44 | 2.2601E-09 | 1.1693E-02 | -7.55 | -41.57 | 4.1602E-03 | -21.85 | -40.73 | -11.19 |
| 9 | 1102.7 | 13.55 | 1.0133E-07 | 1.1612E-02 | 26.84 | -33.44 | 4.1650E-03 | -20.22 | -39.02 | -9.22 |
| 10 | 1244.7 | 2.33 | 1.5952E-08 | 1.1847E-02 | 5.55 | -28.10 | 4.1663E-03 | -20.41 | -39.35 | -9.71 |
| 11 | 1306.3 | 6.80 | 5.7463E-08 | 1.1854E-02 | 6.13 | -27.47 | 4.1637E-03 | -21.01 | -39.94 | -10.32 |
| 12 | 1336.9 | 2.14 | 1.4527E-08 | 1.1840E-02 | 4.99 | -28.67 | 4.1659E-03 | -20.51 | -39.45 | -9.80 |
| 13 | 1376.1 | 1.26 | 9.0473E-09 | 1.1860E-02 | 6.06 | -26.95 | 4.1670E-03 | -20.24 | -39.19 | -9.54 |
| 14 | 1651.7 | 3.44 | 3.4841E-08 | 1.1669E-02 | 7.38 | -26.20 | 4.1658E-03 | -20.54 | -39.48 | -9.83 |
| 15 | 1695.0 | 0.46 | 3.7478E-09 | 1.1663E-02 | 5.89 | -26.70 | 4.1646E-03 | -20.80 | -39.74 | -10.10 |

COPIE CERTIFIEE
CONFORME DES DONNEES
ET FOURNIES ORIGINAUX

Stable Isotope CF Analysis Results

| | |
|--------------------------------|--------------------------------------|
| File: DATA_009.raw | Acquisition Date: 23-7-2006 12:24:58 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1604.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Co2 stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run index: |
| Description: test de stabilite | |

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.65 | 122.7 | 1.1782E-02 | 4.2529E-03 |
| 2 | 4.65 | 182.7 | 1.1781E-02 | 4.2530E-03 |
| 3 | 4.64 | 242.7 | 1.1781E-02 | 4.2527E-03 |
| 20 | 4.63 | 2423.5 | 1.1780E-02 | 4.2524E-03 |
| 21 | 4.64 | 2483.5 | 1.1779E-02 | 4.2523E-03 |
| 22 | 4.69 | 2533.6 | 1.1779E-02 | 4.2526E-03 |

Mean: 1.1780E-02 4.2526E-03
Std Dev of fit (%): 0.05 0.04

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O wrt SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|--------------------|
| 1 | 789.1 | 0.52 | 2.2447E-09 | 1.1884E-02 | 8.77 | -24.82 | 4.1716E-03 | -19.06 | -38.03 | -8.35 |
| 2 | 795.5 | 4.66 | 1.9124E-08 | 1.1861E-02 | 8.89 | -26.71 | 4.1658E-03 | -20.42 | -39.36 | -9.71 |
| 3 | 814.6 | 0.41 | 1.4779E-09 | 1.1810E-02 | 2.52 | -31.25 | 4.1691E-03 | -19.65 | -38.59 | -8.92 |
| 4 | 827.6 | 1.71 | 6.4470E-09 | 1.1884E-02 | 8.77 | -24.78 | 4.1672E-03 | -20.09 | -39.04 | -9.38 |
| 5 | 867.8 | 6.32 | 2.5521E-08 | 1.1830E-02 | 4.19 | -29.49 | 4.1645E-03 | -20.72 | -39.65 | -10.01 |
| 6 | 875.5 | 0.95 | 4.5353E-09 | 1.1798E-02 | 1.46 | -32.30 | 4.1643E-03 | -20.77 | -39.70 | -10.06 |
| 7 | 898.8 | 0.40 | 1.3877E-09 | 1.2041E-02 | 22.09 | -11.08 | 4.1744E-03 | -18.39 | -37.40 | -7.69 |
| 8 | 902.9 | 0.88 | 3.9104E-09 | 1.1718E-02 | -5.25 | -39.23 | 4.1638E-03 | -20.90 | -39.80 | -10.17 |
| 9 | 1114.4 | 0.01 | 1.2401E-09 | 1.1803E-02 | 1.18 | -35.75 | 4.1640E-03 | -20.66 | -39.57 | -9.93 |
| 10 | 1119.5 | 0.61 | 3.5949E-09 | 1.1758E-02 | -1.68 | -35.75 | 4.1640E-03 | -20.66 | -39.57 | -9.93 |
| 11 | 1144.2 | 0.38 | 2.2889E-09 | 1.1919E-02 | 11.80 | -21.65 | 4.1674E-03 | -20.03 | -38.99 | -9.33 |
| 12 | 1243.9 | 0.54 | 3.4381E-09 | 1.1869E-02 | 7.50 | -26.09 | 4.1673E-03 | -20.06 | -39.01 | -9.35 |
| 13 | 1305.1 | 5.27 | 4.1115E-08 | 1.1839E-02 | 4.96 | -28.89 | 4.1641E-03 | -20.83 | -39.76 | -10.12 |
| 14 | 1337.6 | 2.33 | 1.6520E-08 | 1.1795E-02 | 1.29 | -32.48 | 4.1646E-03 | -20.70 | -39.62 | -9.99 |
| 15 | 1378.0 | 0.52 | 3.5830E-09 | 1.1850E-02 | 5.92 | -27.89 | 4.1621E-03 | -21.29 | -40.21 | -10.59 |
| 16 | 1651.9 | 3.02 | 3.0778E-08 | 1.1889E-02 | 7.51 | -26.07 | 4.1652E-03 | -20.57 | -39.51 | -9.87 |

COPIE CERTIFIEE
CONFORME DES DONNEES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_010.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 23-7-2006 13:11:34
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--|-------|--------------------|--------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Label: Value: | | | | | |
| Ratio 1: 13C | | -34.5 | Label: Value: | wrt: | |
| Ratio 2: 18O | | -19.3 | delta 45 | -32.93 | PDB |
| | | | delta 46 | -19.35 | PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.72 | 122.7 | 1.1780E-02 | 4.2525E-03 |
| 2 | 4.73 | 182.7 | 1.1779E-02 | 4.2528E-03 |
| 3 | 4.73 | 242.7 | 1.1780E-02 | 4.2527E-03 |
| 10 | 4.71 | 2423.6 | 1.1780E-02 | 4.2528E-03 |
| 11 | 4.67 | 2483.6 | 1.1779E-02 | 4.2528E-03 |
| 12 | 4.72 | 2533.5 | 1.1780E-02 | 4.2532E-03 |

Mean: 1.1780E-02 4.2528E-03
Std Dev of fit (%): 0.01 0.05

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 852.5 | 0.60 | 2.8614E-09 | 1.1778E-02 | -0.34 | -34.20 | 4.1685E-03 | -19.82 | -38.76 | -9.09 |
| 2 | 866.6 | 3.42 | 1.3279E-08 | 1.1822E-02 | 3.57 | -30.13 | 4.1657E-03 | -20.49 | -39.42 | -9.78 |
| 3 | 1473.7 | 3.12 | 2.8980E-08 | 1.1688E-02 | 9.16 | -24.36 | 4.1660E-03 | -20.42 | -39.37 | -9.72 |
| 4 | 1538.4 | 0.32 | 2.5991E-09 | 1.1881E-02 | 8.58 | -25.00 | 4.1705E-03 | -19.35 | -38.31 | -8.64 |
| 5 | 1762.8 | 1.11 | 1.0322E-08 | 1.1877E-02 | 8.28 | -25.28 | 4.1661E-03 | -20.39 | -39.34 | -9.69 |
| 6 | 1826.5 | 3.57 | 3.7652E-08 | 1.1685E-02 | 8.94 | -24.58 | 4.1660E-03 | -20.65 | -39.59 | -9.95 |

COPIE CERTIFIEE
CONFORME DES DONNEES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_011.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 23-7-2008 13:56:22
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections | |
|-----------------------------------|--------|-----------------|--------|------|------------------------------|--|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None | |
| Gas: CO2 Uncalibrated CO2 | | | | | | |
| Ratio type: Elemental | | | | | | |
| Deconvolution: Craig | | | | | | |
| Elemental delta | | Molecular delta | | | | |
| Label: | Value: | Label: | Value: | wrt: | | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.67 | 122.6 | 1.1780E-02 | 4.2527E-03 |
| 2 | 4.67 | 182.7 | 1.1779E-02 | 4.2527E-03 |
| 3 | 4.70 | 242.7 | 1.1779E-02 | 4.2525E-03 |
| 20 | 4.68 | 2423.5 | 1.1780E-02 | 4.2529E-03 |
| 21 | 4.66 | 2483.5 | 1.1779E-02 | 4.2528E-03 |
| 22 | 4.68 | 2533.6 | 1.1779E-02 | 4.2526E-03 |

Mean: 1.1779E-02 4.2527E-03
Std Dev of fit (%): 0.04 0.03

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 45/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 767.1 | 0.51 | 2.1362E-09 | 1.1772E-02 | -0.59 | -34.54 | 4.1791E-03 | -17.30 | -36.28 | -6.54 |
| 2 | 777.6 | 0.97 | 5.1540E-08 | 1.1786E-02 | 0.53 | -33.27 | 4.1642E-03 | -20.81 | -39.73 | -10.10 |
| 3 | 788.3 | 0.72 | 6.4414E-09 | 1.1773E-02 | -0.51 | -34.36 | 4.1669E-03 | -20.18 | -39.11 | -9.46 |
| 4 | 795.2 | 0.60 | 8.5761E-09 | 1.1762E-02 | -1.45 | -35.34 | 4.1674E-03 | -20.05 | -38.98 | -9.32 |
| 5 | 844.5 | 0.33 | 1.5849E-09 | 1.1848E-02 | 5.85 | -27.80 | 4.1678E-03 | -19.95 | -38.90 | -9.24 |
| 6 | 867.3 | 3.50 | 1.6860E-08 | 1.1806E-02 | 2.28 | -31.46 | 4.1648E-03 | -20.87 | -39.60 | -9.96 |
| 7 | 881.4 | 1.50 | 8.4821E-09 | 1.1764E-02 | -1.33 | -35.19 | 4.1658E-03 | -20.43 | -39.35 | -9.71 |
| 8 | 1121.0 | 0.98 | 7.5055E-08 | 1.1842E-02 | 5.32 | -28.35 | 4.1682E-03 | -19.88 | -38.82 | -9.16 |
| 9 | 1233.6 | 7.77 | 7.8328E-09 | 1.1810E-02 | 16.43 | -29.23 | 4.1673E-03 | -20.23 | -39.41 | -9.48 |
| 10 | 1477.9 | 4.31 | 4.3836E-08 | 1.1891E-02 | 9.45 | -24.06 | 4.1642E-03 | -20.80 | -39.74 | -10.10 |
| 11 | 1506.2 | 0.40 | 3.8366E-09 | 1.1839E-02 | 5.07 | -26.60 | 4.1673E-03 | -20.08 | -39.03 | -9.37 |
| 12 | 1540.8 | 0.38 | 3.1958E-09 | 1.1899E-02 | 10.20 | -23.28 | 4.1641E-03 | -20.83 | -39.77 | -10.14 |
| 13 | 1729.4 | 0.78 | 6.8771E-09 | 1.1803E-02 | 2.00 | -31.80 | 4.1706E-03 | -19.30 | -38.25 | -8.57 |
| 14 | 1784.4 | 1.31 | 1.1941E-08 | 1.1879E-02 | 8.47 | -25.07 | 4.1654E-03 | -20.52 | -39.46 | -9.82 |
| 15 | 1789.1 | 0.38 | 3.6492E-09 | 1.1777E-02 | -0.20 | -34.05 | 4.1688E-03 | -19.72 | -38.66 | -8.99 |
| 16 | 1824.1 | 2.33 | 1.8715E-08 | 1.1893E-02 | 9.65 | -23.85 | 4.1651E-03 | -20.59 | -39.53 | -9.89 |

COPIE CERTIFIEE
CONFORME AUX DONNEES
ET FORMAIRES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_012.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilité

Acquisition Date: 23-7-2006 14:41:6
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by:CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.72 | 122.6 | 1.1779E-02 | 4.2525E-03 |
| 2 | 4.73 | 182.6 | 1.1779E-02 | 4.2528E-03 |
| 3 | 4.73 | 242.6 | 1.1779E-02 | 4.2529E-03 |
| 7 | 4.70 | 2423.5 | 1.1779E-02 | 4.2533E-03 |
| 8 | 4.70 | 2483.5 | 1.1776E-02 | 4.2533E-03 |
| 9 | 4.71 | 2533.5 | 1.1779E-02 | 4.2535E-03 |

Mean: 1.1779E-02 4.2531E-03
Std Dev of fit (%): 0.02 0.04

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 867.9 | 2.61 | 1.0631E-08 | 1.1832E-02 | 4.53 | -29.18 | 4.1706E-03 | -19.39 | -38.34 | -8.66 |
| 2 | 1231.3 | 4.31 | 3.2842E-08 | 1.1888E-02 | 9.23 | -24.28 | 4.1652E-03 | -20.66 | -39.60 | -9.97 |
| 3 | 1257.1 | 5.08 | 3.6249E-08 | 1.1881E-02 | 8.64 | -24.89 | 4.1645E-03 | -20.83 | -39.77 | -10.14 |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FICHES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_013.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 23-7-2006 15:25:49
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: J5 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Molecular delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.68 | 122.6 | 1.1779E-02 | 4.2534E-03 |
| 2 | 4.70 | 182.6 | 1.1779E-02 | 4.2535E-03 |
| 3 | 4.69 | 242.6 | 1.1779E-02 | 4.2535E-03 |
| 12 | 4.66 | 2423.5 | 1.1779E-02 | 4.2529E-03 |
| 13 | 4.63 | 2483.6 | 1.1778E-02 | 4.2525E-03 |
| 14 | 4.66 | 2533.6 | 1.1777E-02 | 4.2526E-03 |

Mean: 1.1779E-02 4.2531E-03
Std Dev of fit (%): 0.04 0.04

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|----------------------|
| 1 | 866.2 | 1.96 | 7.6654E-09 | 1.1831E-02 | 4.45 | -29.24 | 4.1678E-03 | -20.10 | -39.05 | -9.39 |
| 2 | 1229.7 | 3.79 | 2.7752E-08 | 1.1867E-02 | 7.54 | -26.03 | 4.1651E-03 | -20.68 | -39.62 | -9.98 |
| 3 | 1254.5 | 3.20 | 2.1431E-08 | 1.1851E-02 | 6.14 | -27.48 | 4.1654E-03 | -20.62 | -39.55 | -9.91 |
| 4 | 1288.0 | 3.37 | 2.4175E-08 | 1.1803E-02 | 2.04 | -31.71 | 4.1656E-03 | -20.56 | -39.49 | -9.85 |
| 5 | 1346.7 | 0.43 | 3.2135E-09 | 1.1773E-02 | -0.51 | -34.36 | 4.1870E-03 | -20.24 | -39.16 | -9.51 |
| 6 | 1418.5 | 0.47 | 5.4655E-09 | 1.1874E-02 | 8.07 | -25.50 | 4.1678E-03 | -20.06 | -39.01 | -9.35 |
| 7 | 1442.3 | 0.40 | 5.2408E-09 | 1.1861E-02 | 7.02 | -26.56 | 4.1645E-03 | -20.83 | -39.77 | -10.13 |
| 8 | 1560.4 | 0.46 | 5.7271E-09 | 1.1850E-02 | 8.01 | -27.64 | 4.1689E-03 | -19.78 | -38.73 | -9.07 |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_014.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 29-7-2006 20:39:4
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Molecular delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 48 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.73 | 122.7 | 1.1777E-02 | 4.2531E-03 |
| 2 | 4.74 | 182.6 | 1.1778E-02 | 4.2531E-03 |
| 3 | 4.75 | 242.6 | 1.1777E-02 | 4.2536E-03 |
| 8 | 4.74 | 2423.6 | 1.1776E-02 | 4.2527E-03 |
| 9 | 4.74 | 2483.5 | 1.1776E-02 | 4.2528E-03 |
| 10 | 4.74 | 2533.5 | 1.1777E-02 | 4.2534E-03 |

Mean: 1.1777E-02 4.2531E-03
Std Dev of fit (%): 0.02 0.08

Sample Data

| Peak No. | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta18O w.r.t. SMOW |
|----------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|----------------------|
| 1 | 866.4 | 3.91 | 1.5807E-08 | 1.1817E-02 | 3.43 | -30.28 | 4.1656E-03 | -20.58 | -39.51 | -9.87 |
| 2 | 1228.5 | 3.95 | 2.7207E-08 | 1.1938E-02 | 13.65 | -19.73 | 4.1657E-03 | -20.58 | -39.51 | -9.87 |
| 3 | 1300.9 | 2.96 | 1.9556E-08 | 1.1782E-02 | 0.42 | -33.39 | 4.1663E-03 | -20.41 | -39.33 | -9.69 |
| 4 | 1471.8 | 2.49 | 2.2274E-08 | 1.1980E-02 | 17.27 | -16.00 | 4.1675E-03 | -20.13 | -39.10 | -9.45 |

COPIE CERTIFIEE
CONFORME DES DONNEES
ETIQUETTES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_001.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 23-7-2008 9:28:35
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.60 | 42.6 | 1.1783E-02 | 4.2462E-03 |
| 2 | 4.59 | 102.6 | 1.1783E-02 | 4.2479E-03 |
| 3 | 4.60 | 162.7 | 1.1782E-02 | 4.2478E-03 |
| 4 | 4.59 | 222.7 | 1.1782E-02 | 4.2475E-03 |
| 5 | 4.59 | 282.7 | 1.1781E-02 | 4.2478E-03 |
| 6 | 4.60 | 342.7 | 1.1781E-02 | 4.2476E-03 |
| 7 | 4.63 | 402.7 | 1.1780E-02 | 4.2462E-03 |
| 8 | 4.66 | 462.7 | 1.1780E-02 | 4.2465E-03 |
| 9 | 4.66 | 522.7 | 1.1760E-02 | 4.2467E-03 |
| 10 | 4.66 | 582.7 | 1.1780E-02 | 4.2491E-03 |

Mean: 1.1781E-02 4.2481E-03
Std Dev of fit (%): 0.03 0.09

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMELLES ORIGINAUX

Stable Isotope CF Analysis Results

| | |
|--------------------------------|-------------------------------------|
| File: DATA_002.raw | Acquisition Date: 23-7-2006 9:39:43 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1604.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Co2.stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: test de stabilite | |

| Reference standard | Corrections |
|-----------------------------------|-------------------------------|
| Species: CO2 by CF (uncalibrated) | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | |
| Ratio type: Elemental | |
| Deconvolution: Craig | |
| Elemental delta | Molecular delta |
| Label: Value: | Label: Value: wrt: |
| Ratio 1: 13C -34.5 | delta 45 -32.93 PDB |
| Ratio 2: 18O -19.3 | delta 46 -19.35 PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.65 | 42.5 | 1.1781E-02 | 4.2496E-03 |
| 2 | 4.65 | 102.5 | 1.1781E-02 | 4.2498E-03 |
| 3 | 4.65 | 162.5 | 1.1781E-02 | 4.2500E-03 |
| 4 | 4.65 | 222.5 | 1.1781E-02 | 4.2500E-03 |
| 5 | 4.65 | 282.6 | 1.1781E-02 | 4.2501E-03 |
| 6 | 4.63 | 342.6 | 1.1781E-02 | 4.2500E-03 |
| 7 | 4.62 | 402.6 | 1.1781E-02 | 4.2501E-03 |
| 8 | 4.61 | 462.6 | 1.1780E-02 | 4.2497E-03 |
| 9 | 4.63 | 522.7 | 1.1780E-02 | 4.2496E-03 |
| 10 | 4.62 | 582.8 | 1.1780E-02 | 4.2495E-03 |

Mean: 1.1781E-02 4.2498E-03
Std Dev of fit (%): 0.02 0.06

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

COPIE CERTIFIÉE
CONFORMÉ À L'ÉTAT DES LIEUX
ET D'APRÈS LES DONNÉES

Stable Isotope CF Analysis Results

| | |
|--------------------------------|-------------------------------------|
| File: DATA_003.raw | Acquisition Date: 23-7-2006 9:50:48 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1604.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Cc2.stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: test de stabilité | |

| Reference standard | Corrections |
|-----------------------------------|-------------------------------|
| Species: CO2 by CF (uncalibrated) | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | |
| Ratio type: Elemental | |
| Deconvolution: Craig | |
| Elemental delta | Molecular delta |
| Label: Value: | Label: Value: wrt: |
| Ratio 1: 13C -34.5 | delta 45 -32.93 PDB |
| Ratio 2: 18O -19.3 | delta 46 -19.35 PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.83 | 42.6 | 1.1780E-02 | 4.2499E-03 |
| 2 | 4.83 | 102.6 | 1.1780E-02 | 4.2497E-03 |
| 3 | 4.82 | 162.6 | 1.1780E-02 | 4.2495E-03 |
| 4 | 4.84 | 222.6 | 1.1780E-02 | 4.2496E-03 |
| 5 | 4.86 | 282.7 | 1.1780E-02 | 4.2496E-03 |
| 6 | 4.68 | 342.6 | 1.1779E-02 | 4.2499E-03 |
| 7 | 4.71 | 402.6 | 1.1779E-02 | 4.2501E-03 |
| 8 | 4.70 | 462.7 | 1.1780E-02 | 4.2504E-03 |
| 9 | 4.71 | 522.7 | 1.1780E-02 | 4.2507E-03 |
| 10 | 4.70 | 582.7 | 1.1781E-02 | 4.2512E-03 |

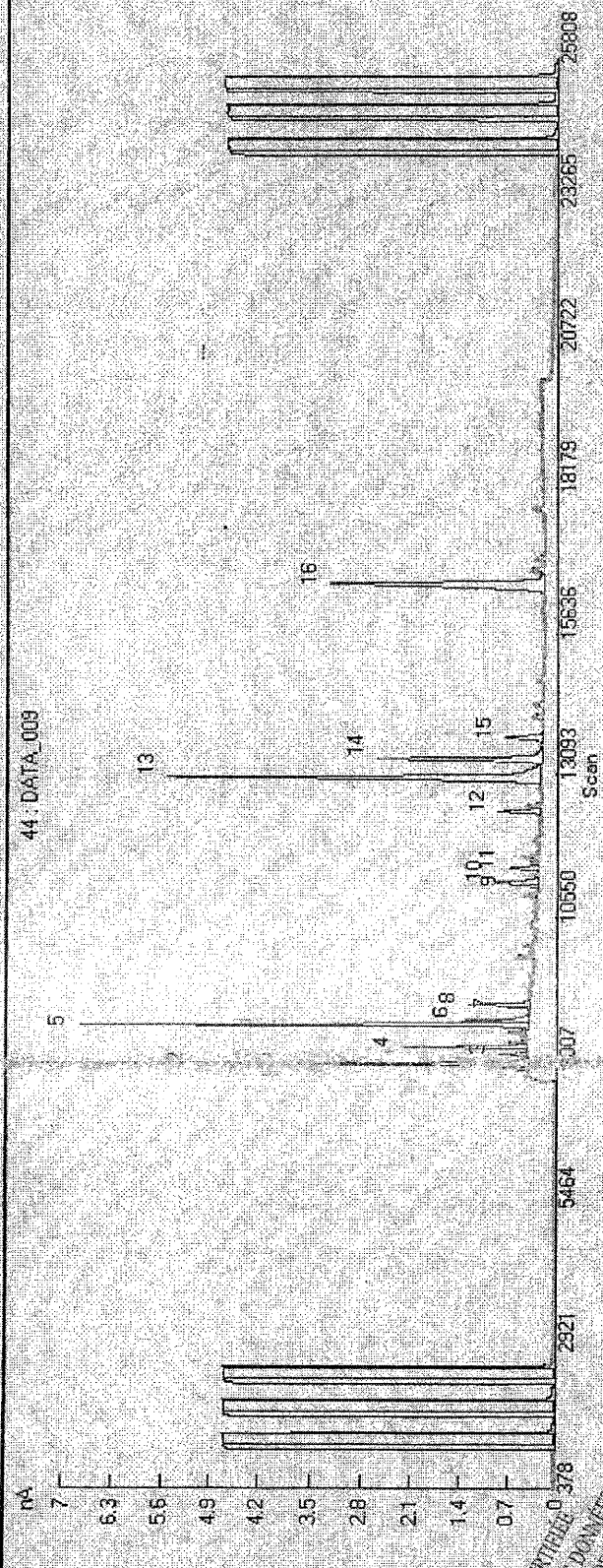
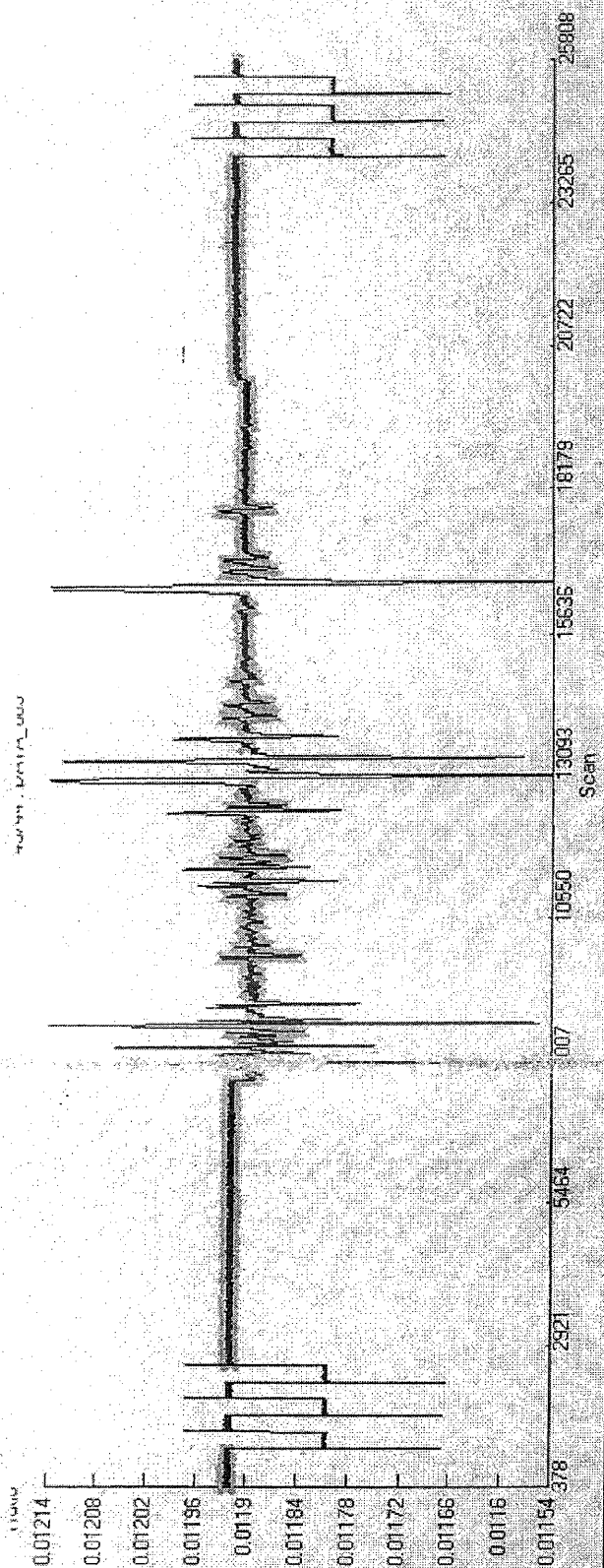
Mean: 1.1780E-02 4.2500E-03
Std Dev of fit (%): 0.04 0.08

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

160



CONFORME DES DONNÉES
ET RÉGULIÈRES ORIGINELLES

GDC01030

Stable Isotope CF Analysis Results

File: DATA_009.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 8/4/06 12:24
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|-----------------|--------|-----------------|--------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| | Elemental delta | | Molecular delta | | |
| | Label: | Value: | Label: | Value: | wrt: |
| Ratio 1: 13C | | -34.5 | delta 45 | -32.93 | PDB |
| Ratio 2: 18O | | -19.3 | delta 46 | -19.35 | PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.65 | 122.6 | 1.1776E-02 | 4.2526E-03 |
| 2 | 4.67 | 182.6 | 1.1776E-02 | 4.2527E-03 |
| 3 | 4.64 | 242.6 | 1.1776E-02 | 4.2528E-03 |
| 8 | 4.62 | 2423.5 | 1.1775E-02 | 4.2525E-03 |
| 9 | 4.60 | 2483.5 | 1.1775E-02 | 4.2521E-03 |
| 10 | 4.63 | 2533.6 | 1.1775E-02 | 4.2523E-03 |

Mean: 1.1776E-02 4.2525E-03
Std Dev of fit (%): 0.01 0.04

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 870.9 | 4.19 | 1.7923E-08 | 1.1817E-02 | 3.47 | -30.23 | 4.1836E-03 | -20.90 | -39.82 | -10.19 |
| 2 | 1242.1 | 4.25 | 3.2043E-08 | 1.1937E-02 | 13.70 | -19.66 | 4.1645E-03 | -20.70 | -39.65 | -10.01 |
| 3 | 1316.3 | 3.24 | 2.3868E-08 | 1.1780E-02 | 0.38 | -33.44 | 4.1646E-03 | -20.68 | -39.60 | -9.97 |
| 4 | 1491.1 | 2.72 | 2.7260E-08 | 1.1917E-02 | 17.04 | -16.21 | 4.1641E-03 | -20.79 | -39.75 | -10.11 |

Stable Isotope CF Analysis Results

| | |
|--------------------------------|--------------------------------|
| File: DATA_010.raw | Acquisition Date: 8/4/05 17:03 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1504.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Co2.stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: test de stabilite | |

| Reference standard | Corrections |
|---|------------------------------|
| Species: CO2 by CF (uncalibrated) | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | |
| Ratio type: Elemental | |
| Deconvolution: Craig | |
| Elemental delta | |
| Label: Value: | |
| Ratio 1: 13C -34.5 delta 45 -32.93 wrt: PDB | |
| Ratio 2: 18O -19.3 delta 46 -19.35 PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.68 | 122.7 | 1.1774E-02 | 4.2511E-03 |
| 2 | 4.67 | 182.7 | 1.1773E-02 | 4.2509E-03 |
| 3 | 4.67 | 242.7 | 1.1773E-02 | 4.2504E-03 |
| 19 | 4.64 | 2423.6 | 1.1772E-02 | 4.2507E-03 |
| 20 | 4.60 | 2483.6 | 1.1772E-02 | 4.2508E-03 |
| 21 | 4.64 | 2533.5 | 1.1773E-02 | 4.2509E-03 |

Mean: 1.1773E-02 4.2509E-03
Std Dev of fit (%): 0.03 0.07

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 788.8 | 0.32 | 1.0307E-09 | 1.2081E-02 | 26.19 | -6.35 | 4.1735E-03 | -18.20 | -37.22 | -7.51 |
| 2 | 791.1 | 0.32 | 1.3212E-09 | 1.1813E-02 | 3.39 | -30.31 | 4.1627E-03 | -20.74 | -39.67 | -10.03 |
| 3 | 799.6 | 13.34 | 5.3484E-08 | 1.1874E-02 | 8.63 | -24.87 | 4.1592E-03 | -21.56 | -40.48 | -10.87 |
| 4 | 872.1 | 5.14 | 2.0590E-08 | 1.1809E-02 | 3.05 | -30.66 | 4.1621E-03 | -20.87 | -39.80 | -10.17 |
| 5 | 880.7 | 2.17 | 1.0421E-08 | 1.1838E-02 | 5.58 | -28.07 | 4.1617E-03 | -20.97 | -39.90 | -10.27 |
| 6 | 1052.2 | 0.43 | 3.7599E-09 | 1.1831E-02 | 4.97 | -28.69 | 4.1644E-03 | -20.32 | -39.26 | -9.61 |
| 7 | 1130.5 | 0.30 | 2.4675E-09 | 1.1851E-02 | 6.68 | -26.95 | 4.1670E-03 | -19.71 | -38.67 | -9.00 |
| 8 | 1158.2 | 0.52 | 3.3251E-09 | 1.1855E-02 | 6.80 | -23.71 | 4.1640E-03 | -20.42 | -38.37 | -9.72 |
| 9 | 1179.8 | 0.44 | 2.8313E-09 | 1.1837E-02 | 2.43 | -28.24 | 4.1690E-03 | -19.24 | -36.20 | -8.52 |
| 10 | 1260.4 | 2.32 | 1.7351E-08 | 1.1838E-02 | 5.50 | -28.13 | 4.1624E-03 | -20.79 | -39.73 | -10.09 |
| 11 | 1322.6 | 6.69 | 5.6884E-08 | 1.1839E-02 | 5.65 | -27.96 | 4.1609E-03 | -21.15 | -40.08 | -10.45 |
| 12 | 1353.7 | 1.80 | 1.2943E-08 | 1.1820E-02 | 3.96 | -29.73 | 4.1638E-03 | -20.46 | -39.39 | -9.75 |
| 13 | 1394.5 | 1.62 | 1.1764E-08 | 1.1833E-02 | 5.11 | -28.53 | 4.1621E-03 | -20.87 | -39.80 | -10.17 |
| 14 | 1674.5 | 3.11 | 3.1706E-08 | 1.1859E-02 | 7.31 | -26.26 | 4.1619E-03 | -20.91 | -39.84 | -10.21 |
| 15 | 1717.8 | 0.39 | 3.1228E-09 | 1.1853E-02 | 6.84 | -26.74 | 4.1622E-03 | -20.86 | -39.79 | -10.16 |

COPIES
CONTIENE LES DONNEES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

| | |
|--------------------------------|--------------------------------|
| File: DATA_011.raw | Acquisition Date: 8/4/06 17:48 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1804.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Co2 stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: test de stabilit  | |

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Molecular delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.81 | 122.7 | 1.1772E-02 | 4.2501E-03 |
| 2 | 4.63 | 182.8 | 1.1771E-02 | 4.2504E-03 |
| 3 | 4.62 | 242.8 | 1.1771E-02 | 4.2502E-03 |
| 21 | 4.63 | 2423.6 | 1.1772E-02 | 4.2505E-03 |
| 22 | 4.63 | 2483.6 | 1.1772E-02 | 4.2505E-03 |
| 23 | 4.67 | 2533.6 | 1.1772E-02 | 4.2510E-03 |

Mean: 1.1772E-02 4.2505E-03
Std Dev of fit (%): 0.03 0.05

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 779.2 | 0.32 | 5.0274E-09 | 1.1787E-02 | 1.33 | -32.48 | 4.1678E-03 | -19.45 | -38.40 | -8.72 |
| 2 | 797.1 | 1.70 | 1.7500E-08 | 1.1827E-02 | 4.67 | -29.01 | 4.1650E-03 | -20.12 | -39.06 | -9.40 |
| 3 | 817.2 | 0.53 | 6.0358E-09 | 1.1792E-02 | 1.75 | -32.03 | 4.1666E-03 | -19.73 | -38.67 | -9.00 |
| 4 | 830.4 | 0.81 | 5.7831E-09 | 1.1849E-02 | 8.59 | -27.04 | 4.1688E-03 | -19.69 | -38.64 | -8.97 |
| 5 | 838.9 | 1.37 | 9.0887E-09 | 1.1760E-02 | -0.99 | -34.85 | 4.1637E-03 | -20.41 | -39.34 | -9.69 |
| 6 | 850.4 | 0.42 | 3.9066E-09 | 1.1768E-02 | -0.30 | -34.15 | 4.1652E-03 | -20.05 | -38.98 | -9.33 |
| 7 | 858.5 | 1.04 | 7.8086E-09 | 1.1756E-02 | -1.37 | -35.24 | 4.1643E-03 | -20.28 | -39.20 | -9.55 |
| 8 | 871.3 | 0.74 | 1.8804E-08 | 1.1820E-02 | 4.68 | -29.51 | 4.1633E-03 | -20.19 | -39.42 | -9.78 |
| 9 | 881.1 | 3.08 | 2.2650E-08 | 1.1827E-02 | 4.66 | -28.61 | 4.1622E-03 | -20.77 | -39.70 | -10.07 |
| 10 | 909.2 | 2.87 | 1.4120E-08 | 1.1751E-02 | -0.92 | -34.77 | 4.1637E-03 | -20.41 | -39.33 | -9.69 |
| 11 | 995.8 | 0.92 | 5.0720E-09 | 1.1715E-02 | -4.81 | -38.78 | 4.1625E-03 | -20.70 | -39.61 | -9.97 |
| 12 | 1106.6 | 0.35 | 2.2759E-09 | 1.1636E-02 | -11.51 | -45.69 | 4.1617E-03 | -20.87 | -39.77 | -10.14 |
| 13 | 1257.7 | 0.42 | 2.6334E-09 | 1.1859E-02 | 7.38 | -26.20 | 4.1643E-03 | -20.27 | -39.21 | -9.56 |
| 14 | 1318.3 | 3.95 | 3.0575E-08 | 1.1832E-02 | 5.11 | -28.54 | 4.1622E-03 | -20.77 | -39.70 | -10.06 |
| 15 | 1352.2 | 2.07 | 1.3795E-08 | 1.1792E-02 | 1.72 | -32.04 | 4.1629E-03 | -20.59 | -39.52 | -9.88 |
| 16 | 1391.5 | 0.39 | 3.0840E-09 | 1.1832E-02 | 5.12 | -28.54 | 4.1630E-03 | -20.57 | -39.50 | -9.86 |
| 17 | 1671.1 | 2.35 | 2.2808E-08 | 1.1863E-02 | 7.79 | -25.78 | 4.1634E-03 | -20.47 | -39.42 | -9.77 |

COPIE CERTIFIEE
DES FICHES DES DONNEES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

| | |
|--------------------------------|--------------------------------|
| File: DATA_012.raw | Acquisition Date: 8/4/00 16:33 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1604.spl | Injection Volume: 0 |
| Liner: 1 | Bottle: |
| MS file: Co2 stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: test de stabilite | |

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|-----------------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated: CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | Molecular delta | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.67 | 122.7 | 1.1773E-02 | 4.2515E-03 |
| 2 | 4.65 | 182.5 | 1.1773E-02 | 4.2517E-03 |
| 3 | 4.65 | 242.7 | 1.1773E-02 | 4.2515E-03 |
| 9 | 4.65 | 2423.8 | 1.1774E-02 | 4.2518E-03 |
| 10 | 4.62 | 2483.5 | 1.1774E-02 | 4.2518E-03 |
| 11 | 4.67 | 2533.5 | 1.1774E-02 | 4.2522E-03 |

Mean: 1.1773E-02 4.2517E-03
Std Dev of fit (%): 0.01 0.04

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 855.5 | 0.52 | 2.5179E-09 | 1.1760E-02 | -1.13 | -34.99 | 4.1649E-03 | -20.42 | -39.34 | -9.70 |
| 2 | 869.7 | 1.76 | 7.4253E-09 | 1.1814E-02 | 3.43 | -30.27 | 4.1632E-03 | -20.82 | -39.74 | -10.11 |
| 3 | 1489.9 | 2.77 | 2.5577E-09 | 1.1881E-02 | 9.10 | -24.41 | 4.1628E-03 | -20.92 | -39.66 | -10.23 |
| 4 | 1781.9 | 0.89 | 8.7577E-09 | 1.1866E-02 | 7.88 | -25.67 | 4.1635E-03 | -20.75 | -39.69 | -10.06 |
| 5 | 1844.0 | 3.10 | 3.0652E-08 | 1.1875E-02 | 8.66 | -24.86 | 4.1619E-03 | -21.12 | -40.05 | -10.43 |

Stable Isotope CF Analysis Results

| | |
|--------------------------------|--------------------------------|
| File: DATA_013.raw | Acquisition Date: 8/4/06 19:18 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1804.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Co2 stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: test de stabilite | |

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.83 | 122.8 | 1.1774E-02 | 4.2514E-03 |
| 2 | 4.85 | 182.7 | 1.1773E-02 | 4.2514E-03 |
| 3 | 4.88 | 242.8 | 1.1773E-02 | 4.2517E-03 |
| 12 | 4.65 | 2423.6 | 1.1775E-02 | 4.2525E-03 |
| 13 | 4.62 | 2483.6 | 1.1775E-02 | 4.2527E-03 |
| 14 | 4.68 | 2533.5 | 1.1775E-02 | 4.2533E-03 |

Mean: 1.1774E-02 4.2522E-03
Std Dev of fit (%): 0.03 0.07

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 46/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 776.9 | 4.08 | 2.2482E-08 | 1.1781E-02 | 0.57 | -33.23 | 4.1649E-03 | -20.52 | -39.44 | -9.80 |
| 2 | 870.2 | 2.62 | 1.1281E-08 | 1.1812E-02 | 3.20 | -30.50 | 4.1840E-03 | -20.75 | -39.67 | -10.04 |
| 3 | 884.4 | 0.65 | 4.7715E-09 | 1.1746E-02 | -2.37 | -36.26 | 4.1829E-03 | -21.00 | -39.91 | -10.29 |
| 4 | 1247.7 | 0.62 | 3.9004E-09 | 1.1818E-02 | 3.72 | -29.98 | 4.1649E-03 | -20.52 | -39.46 | -9.81 |
| 5 | 1490.0 | 2.53 | 2.2980E-08 | 1.1887E-02 | 9.58 | -23.92 | 4.1839E-03 | -20.75 | -39.69 | -10.06 |
| 6 | 1746.9 | 0.35 | 3.3134E-09 | 1.1832E-02 | 4.91 | -28.84 | 4.1780E-03 | -17.91 | -36.89 | -7.17 |
| 7 | 1780.1 | 0.45 | 4.0461E-09 | 1.1884E-02 | 9.31 | -24.22 | 4.1688E-03 | -20.07 | -39.02 | -9.37 |
| 8 | 1838.5 | 0.89 | 7.7476E-09 | 1.1882E-02 | 9.17 | -24.35 | 4.1647E-03 | -20.57 | -39.51 | -9.87 |

COPIE CERTIFIÉE
CONFORME DES DOCUMENTS
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_014.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilité

Acquisition Date: 8/4/06 20:02
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections | |
|-----------------------------------|--------|-----------------|--|-----------------|------------------------------|------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None | |
| Gas: CO2 Uncalibrated CO2 | | | | | | |
| Ratio type: Elemental | | | | | | |
| Deconvolution: Craig | | | | | | |
| | | Elemental delta | | Molecular delta | | |
| | Label: | Value: | | Label: | Value: | wrt: |
| Ratio 1: | 13C | -34.5 | | delta 45 | -32.93 | PDB |
| Ratio 2: | 18O | -19.3 | | delta 46 | -19.35 | PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.65 | 122.7 | 1.1776E-02 | 4.2531E-03 |
| 2 | 4.65 | 182.7 | 1.1776E-02 | 4.2531E-03 |
| 3 | 4.65 | 242.7 | 1.1776E-02 | 4.2528E-03 |
| 7 | 4.62 | 2423.6 | 1.1774E-02 | 4.2522E-03 |
| 8 | 4.58 | 2483.6 | 1.1774E-02 | 4.2518E-03 |
| 9 | 4.63 | 2533.6 | 1.1773E-02 | 4.2521E-03 |

Mean: 1.1775E-02 4.2525E-03
Std Dev of fit (%): 0.03 0.04

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 869.4 | 2.27 | 9.8987E-09 | 1.1819E-02 | 3.72 | -29.98 | 4.1662E-03 | -20.30 | -39.24 | -9.59 |
| 2 | 1240.6 | 2.91 | 2.2334E-08 | 1.1877E-02 | 8.67 | -24.87 | 4.1653E-03 | -20.52 | -39.47 | -9.83 |
| 3 | 1267.3 | 3.31 | 2.3552E-08 | 1.1868E-02 | 9.59 | -23.91 | 4.1645E-03 | -20.71 | -39.65 | -10.02 |

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINALS

167

Stable Isotope CF Analysis Results

| | |
|--------------------------------|--------------------------------|
| File: DATA_015.raw | Acquisition Date: 8/4/08 20:47 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1804.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Co2 stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: test de stabilite | |

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|-----------------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | Molecular delta | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.68 | 122.6 | 1.1774E-02 | 4.2526E-03 |
| 2 | 4.70 | 182.6 | 1.1775E-02 | 4.2533E-03 |
| 3 | 4.71 | 242.6 | 1.1775E-02 | 4.2538E-03 |
| 11 | 4.66 | 2423.5 | 1.1773E-02 | 4.2524E-03 |
| 12 | 4.68 | 2483.6 | 1.1773E-02 | 4.2524E-03 |
| 13 | 4.70 | 2533.5 | 1.1773E-02 | 4.2530E-03 |

Mean: 1.1774E-02 4.2529E-03
Std Dev of fit (%): 0.05 0.12

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 870.3 | 3.34 | 1.4409E-08 | 1.1816E-02 | 3.56 | -30.14 | 4.1845E-03 | -20.80 | -39.73 | -10.09 |
| 2 | 1241.4 | 3.84 | 2.8786E-08 | 1.1862E-02 | 7.49 | -28.08 | 4.1845E-03 | -20.78 | -39.72 | -10.08 |
| 3 | 1266.6 | 2.73 | 1.8545E-08 | 1.1845E-02 | 8.07 | -27.55 | 4.1850E-03 | -20.67 | -39.61 | -9.87 |
| 4 | 1301.6 | 3.69 | 2.7446E-08 | 1.1799E-02 | 2.16 | -31.58 | 4.1648E-03 | -20.72 | -39.65 | -10.01 |
| 5 | 1360.9 | 0.48 | 3.5101E-09 | 1.1780E-02 | 0.50 | -33.30 | 4.1849E-03 | -20.69 | -39.61 | -9.97 |
| 6 | 1433.3 | 0.81 | 8.2517E-09 | 1.1862E-02 | 7.52 | -26.05 | 4.1857E-03 | -20.51 | -39.45 | -9.81 |
| 7 | 1580.7 | 0.79 | 9.1617E-09 | 1.1842E-02 | 5.75 | -27.88 | 4.1853E-03 | -20.59 | -39.63 | -9.89 |

COPIE CERTIFIEE
CONFORME DES DONNEES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_016.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 8/4/06 21:32
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JS 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Crag | | | | | |
| Elemental delta | | | | | |
| Molecular delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.67 | 122.5 | 1.1775E-02 | 4.2532E-03 |
| 2 | 4.66 | 182.7 | 1.1775E-02 | 4.2531E-03 |
| 3 | 4.65 | 242.7 | 1.1774E-02 | 4.2530E-03 |
| 8 | 4.62 | 2423.5 | 1.1774E-02 | 4.2528E-03 |
| 9 | 4.60 | 2483.5 | 1.1774E-02 | 4.2524E-03 |
| 10 | 4.65 | 2533.6 | 1.1773E-02 | 4.2526E-03 |

Mean: 1.1774E-02 4.2529E-03
Std Dev of fit (%): 0.04 0.03

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
| 1 | 870.2 | 4.21 | 1.7786E-08 | 1.1818E-02 | -3.69 | -30.01 | 4.1847E-03 | -20.73 | -39.66 | -10.02 |
| 2 | 1242.1 | 4.12 | 3.1178E-08 | 1.1936E-02 | 13.78 | -19.60 | 4.1849E-03 | -20.69 | -39.64 | -10.00 |
| 3 | 1315.8 | 3.17 | 2.2902E-08 | 1.1778E-02 | 0.32 | -33.47 | 4.1843E-03 | -20.81 | -39.73 | -10.10 |
| 4 | 1490.6 | 2.88 | 2.6475E-08 | 1.1975E-02 | 17.09 | -16.17 | 4.1842E-03 | -20.84 | -39.80 | -10.16 |

05/05/2007 13:09

Results for CO2 by CF (uncalibrated)(Fnt) [Printout]

CORRIGEE
BIEN CONFORME DES DONNEES
ET POUR LES ORIGINAUX

GDC01038

169

Stable Isotope CF Analysis Results

File: DATA_005.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 6/4/06 11:08
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections | |
|-----------------------------------|--------|----------|--------|------|------------------------------|--|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None | |
| Gas: CO2 Uncalibrated CO2 | | | | | | |
| Ratio type: Elemental | | | | | | |
| Deconvolution: Craig | | | | | | |
| Elemental delta | | | | | Molecular delta | |
| Label: | Value: | Label: | Value: | wrt: | | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.64 | 42.5 | 1.1779E-02 | 4.2531E-03 |
| 2 | 4.63 | 102.5 | 1.1779E-02 | 4.2533E-03 |
| 3 | 4.64 | 162.6 | 1.1779E-02 | 4.2536E-03 |
| 4 | 4.64 | 222.6 | 1.1779E-02 | 4.2535E-03 |
| 5 | 4.63 | 282.6 | 1.1779E-02 | 4.2539E-03 |
| 6 | 4.62 | 342.6 | 1.1780E-02 | 4.2540E-03 |
| 7 | 4.62 | 402.7 | 1.1780E-02 | 4.2539E-03 |
| 8 | 4.63 | 462.7 | 1.1779E-02 | 4.2540E-03 |
| 9 | 4.61 | 522.7 | 1.1780E-02 | 4.2537E-03 |
| 10 | 4.60 | 582.7 | 1.1779E-02 | 4.2535E-03 |

Mean: 1.1779E-02 4.2537E-03
Std Dev of fit (%): 0.02 0.06

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

Stable Isotope CF Analysis Results

File: DATA_004.raw
Project: controle2007.PRO
Sample list: 1804.spl
Line: 1
MS file: Co2 stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 8/4/08 10:57
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|-----------------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | Molecular delta | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 48 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.62 | 42.5 | 1.1781E-02 | 4.2542E-03 |
| 2 | 4.60 | 102.5 | 1.1781E-02 | 4.2541E-03 |
| 3 | 4.59 | 162.8 | 1.1781E-02 | 4.2538E-03 |
| 4 | 4.59 | 222.6 | 1.1781E-02 | 4.2537E-03 |
| 5 | 4.58 | 282.7 | 1.1781E-02 | 4.2534E-03 |
| 6 | 4.58 | 342.7 | 1.1780E-02 | 4.2531E-03 |
| 7 | 4.58 | 402.7 | 1.1780E-02 | 4.2527E-03 |
| 8 | 4.59 | 462.8 | 1.1780E-02 | 4.2527E-03 |
| 9 | 4.60 | 522.8 | 1.1779E-02 | 4.2526E-03 |
| 10 | 4.63 | 582.7 | 1.1778E-02 | 4.2526E-03 |

Mean: 1.1780E-02 4.2533E-03
Std Dev of fit (%): 0.02 0.04

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

05/05/2007 12:26

Results for CO2 by CF (uncalibrated)(Fr1) [Printout]

COPIE CERTIFIEE
CONFORME DES DONNEES
ET FORMULAIRES ORIGINAUX

1 of 1

GDC01040

171

Stable Isotope CF Analysis Results

File: DATA_D03.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2 slab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 8/4/06 10:46
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections | | |
|-----------------------------------|--|--------|--|---------------------------|------------------------------|--|--|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None | | |
| Gas: CO2 Uncalibrated CO2 | | | | | | | |
| Ratio type: Elemental | | | | | | | |
| Deconvolution: Craig | | | | | | | |
| Elemental delta | | | | | | | |
| Label: | | Value: | | wrt: | | | |
| Ratio 1: 13C | | -34.5 | | delta 45 -32.93 PDB | | | |
| Ratio 2: 18O | | -19.3 | | delta 46 -19.35 PDB | | | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.59 | 42.6 | 1.1780E-02 | 4.2524E-03 |
| 2 | 4.62 | 102.5 | 1.1779E-02 | 4.2525E-03 |
| 3 | 4.64 | 162.5 | 1.1779E-02 | 4.2525E-03 |
| 4 | 4.63 | 222.5 | 1.1779E-02 | 4.2528E-03 |
| 5 | 4.64 | 282.6 | 1.1779E-02 | 4.2530E-03 |
| 6 | 4.62 | 342.6 | 1.1779E-02 | 4.2531E-03 |
| 7 | 4.61 | 402.6 | 1.1780E-02 | 4.2533E-03 |
| 8 | 4.62 | 462.6 | 1.1780E-02 | 4.2538E-03 |
| 9 | 4.65 | 522.7 | 1.1780E-02 | 4.2542E-03 |
| 10 | 4.65 | 582.7 | 1.1781E-02 | 4.2546E-03 |

Mean: 1.1780E-02 4.2532E-03
Std Dev of fit (%): 0.04 0.05

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

Stable Isotope CF Analysis Results

| | |
|--------------------------------|--------------------------------|
| File: DATA_002.raw | Acquisition Date: 8/4/06 10:35 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1604.spl | Injection Volume: 0 |
| Line: 1 | Bottle: |
| MS file: Co2 stab | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: Do Nothing | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: test de stabilite | |

| Reference standard | | | | | Corrections | |
|-----------------------------------|--------|----------|--------|------|------------------------------|--|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None | |
| Gas: CO2 Uncalibrated CO2 | | | | | | |
| Ratio type: Elemental | | | | | | |
| Deconvolution: Craig | | | | | | |
| Elemental delta | | | | | | |
| Label: | Value: | Label: | Value: | wrt: | | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.63 | 42.5 | 1.1782E-02 | 4.2528E-03 |
| 2 | 4.63 | 102.5 | 1.1782E-02 | 4.2529E-03 |
| 3 | 4.62 | 162.5 | 1.1782E-02 | 4.2530E-03 |
| 4 | 4.62 | 222.6 | 1.1782E-02 | 4.2529E-03 |
| 5 | 4.62 | 282.6 | 1.1781E-02 | 4.2529E-03 |
| 6 | 4.60 | 342.6 | 1.1781E-02 | 4.2528E-03 |
| 7 | 4.60 | 402.7 | 1.1781E-02 | 4.2528E-03 |
| 8 | 4.59 | 462.7 | 1.1781E-02 | 4.2524E-03 |
| 9 | 4.58 | 522.8 | 1.1780E-02 | 4.2522E-03 |
| 10 | 4.57 | 582.8 | 1.1780E-02 | 4.2518E-03 |

Mean: 1.1781E-02 4.2526E-03
Std Dev of fit (%): 0.02 0.05

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

Stable Isotope CF Analysis Results

File: DATA_001.raw
Project: controle2007.PRO
Sample list: 1604.spl
Line: 1
MS file: Co2.stab
Inlet: GC-combustion
Inlet file: Do Nothing
Sample ID:
Description: test de stabilite

Acquisition Date: 8/4/06 10:24
Weight: 0.00
Injection Volume: 0
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 4.58 | 42.6 | 1.1784E-02 | 4.2520E-03 |
| 2 | 4.58 | 102.6 | 1.1784E-02 | 4.2517E-03 |
| 3 | 4.58 | 162.6 | 1.1783E-02 | 4.2513E-03 |
| 4 | 4.58 | 222.7 | 1.1783E-02 | 4.2511E-03 |
| 5 | 4.59 | 282.7 | 1.1782E-02 | 4.2512E-03 |
| 6 | 4.62 | 342.6 | 1.1782E-02 | 4.2511E-03 |
| 7 | 4.63 | 402.7 | 1.1781E-02 | 4.2513E-03 |
| 8 | 4.63 | 462.7 | 1.1781E-02 | 4.2516E-03 |
| 9 | 4.62 | 522.7 | 1.1781E-02 | 4.2516E-03 |
| 10 | 4.62 | 582.7 | 1.1781E-02 | 4.2518E-03 |

Mean: 1.1782E-02 4.2515E-03
Std Dev of fit (%): 0.03 0.08

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

Stable Isotope CF Analysis Results

File: DATA_008.raw
Project: controle2007.PRO
Sample list: 1704.spl
Line: 15
MS file: M-AN41
Inlet: GC-combustion
Inlet file: M-AN41
Sample ID:
Description: Calibration Mix Cal Acetate 001C

Acquisition Date: 26-6-2006 11:23:11
Weight: 0.00
Injection Volume: 10
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections | |
|-----------------------------------|--------|-----------------|----------|-----------------|------------------------------|--|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None | |
| Gas: CO2 Uncalibrated CO2 | | | | | | |
| Ratio type: Elemental | | | | | | |
| Deconvolution: Craig | | | | | | |
| | | Elemental delta | | Molecular delta | | |
| | Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: | 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: | 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 1.57 | 42.6 | 1.1790E-02 | 4.2594E-03 |
| 2 | 1.57 | 102.6 | 1.1789E-02 | 4.2592E-03 |
| 3 | 5.91 | 162.8 | 1.1792E-02 | 4.2580E-03 |
| 4 | 5.92 | 222.6 | 1.1792E-02 | 4.2579E-03 |
| 5 | 3.98 | 282.6 | 1.1791E-02 | 4.2586E-03 |
| 6 | 3.97 | 342.6 | 1.1791E-02 | 4.2582E-03 |
| 7 | 9.33 | 402.7 | 1.1792E-02 | 4.2571E-03 |
| 8 | 9.32 | 462.6 | 1.1792E-02 | 4.2574E-03 |
| 9 | 1.47 | 522.7 | 1.1788E-02 | 4.2591E-03 |
| 10 | 1.48 | 582.8 | 1.1788E-02 | 4.2588E-03 |

Mean: 1.1790E-02 4.2584E-03
Std Dev of fit (%): 0.13 0.19

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_009.raw Acquisition Date: 28-6-2006 11:35:39
 Project: controle2007.PRO Weight: 0.00
 Sample list: 1704.spl Injection Volume: 10
 Line: 15 Bottle:
 MS file: M-AN41 Type:
 Inlet: GC-combustion Standard:
 Inlet file: M-AN41 Slot Number: JB 251
 Sample ID: Run Index:
 Description: Calibration Mix Cal Acetate 001C

| Reference standard | Corrections |
|-----------------------------------|------------------------------|
| Species: CO2 by CF (uncalibrated) | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | |
| Ratio type: Elemental | |
| Deconvolution: Craig | |
| Elemental delta | |
| Label: Value: | |
| Ratio 1: 13C -34.5 | delta 45 -32.93 wrt: PDB |
| Ratio 2: 18O -19.3 | delta 46 -19.35 wrt: PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 1.48 | 42.6 | 1.1788E-02 | 4.2590E-03 |
| 2 | 1.48 | 102.8 | 1.1788E-02 | 4.2586E-03 |
| 3 | 5.93 | 162.6 | 1.1792E-02 | 4.2581E-03 |
| 4 | 5.93 | 222.6 | 1.1791E-02 | 4.2582E-03 |
| 5 | 3.82 | 282.7 | 1.1791E-02 | 4.2588E-03 |
| 6 | 3.82 | 342.6 | 1.1791E-02 | 4.2587E-03 |
| 7 | 9.68 | 402.7 | 1.1792E-02 | 4.2572E-03 |
| 8 | 9.66 | 462.8 | 1.1792E-02 | 4.2574E-03 |
| 9 | 1.48 | 522.8 | 1.1788E-02 | 4.2592E-03 |
| 10 | 1.49 | 582.8 | 1.1788E-02 | 4.2590E-03 |

Mean: 1.1790E-02 4.2584E-03
 Std Dev of fit (%): 0.15 0.17

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

File: DATA_010.raw
Project: controle2007.PRO
Sample list: 1704.spl
Line: 15
MS file: M-AN41
Inlet: GC-combustion
Inlet file: M-AN41
Sample ID:
Description: Calibration Mix Cal Acetate 0010

Acquisition Date: 26-6-2006 11:47:5
Weight: 0.00
Injection Volume: 10
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | 19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 1.49 | 42.6 | 1.1788E-02 | 4.2585E-03 |
| 2 | 1.49 | 102.6 | 1.1786E-02 | 4.2585E-03 |
| 3 | 5.87 | 162.6 | 1.1791E-02 | 4.2577E-03 |
| 4 | 5.86 | 222.6 | 1.1790E-02 | 4.2572E-03 |
| 5 | 3.80 | 282.6 | 1.1788E-02 | 4.2585E-03 |
| 6 | 3.79 | 342.7 | 1.1788E-02 | 4.2566E-03 |
| 7 | 9.78 | 402.7 | 1.1790E-02 | 4.2558E-03 |
| 8 | 9.79 | 462.7 | 1.1790E-02 | 4.2560E-03 |
| 9 | 1.56 | 522.8 | 1.1787E-02 | 4.2562E-03 |
| 10 | 1.56 | 582.8 | 1.1788E-02 | 4.2556E-03 |

Mean: 1.1789E-02 4.2569E-03
Std Dev of fit (%): 0.12 0.10

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

Stable Isotope CF Analysis Results

| | |
|---|--------------------------------------|
| File: DATA_007.raw | Acquisition Date: 31-7-2006 11:58:36 |
| Project: controle2007.PRO | Weight: 0.00 |
| Sample list: 1704.spl | Injection Volume: 10 |
| Line: 15 | Bottle: |
| MS file: M-AN41 | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: M-AN41 | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: Calibration Mix Cal Acetate 001C | |

| Reference standard | Corrections |
|-----------------------------------|-------------------------------|
| Species: CO2 by CF (uncalibrated) | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | |
| Ratio type: Elemental | |
| Deconvolution: Craig | |
| Elemental delta | Molecular delta |
| Label: Value: | Label: Value: wrt: |
| Ratio 1: 13C -34.5 | delta 45 -32.93 PDB |
| Ratio 2: 18O -19.3 | delta 46 -19.35 PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 1.66 | 42.7 | 1.1777E-02 | 4.2508E-03 |
| 2 | 1.67 | 102.7 | 1.1777E-02 | 4.2508E-03 |
| 3 | 6.78 | 182.6 | 1.1778E-02 | 4.2495E-03 |
| 4 | 6.76 | 222.6 | 1.1778E-02 | 4.2497E-03 |
| 5 | 4.06 | 282.7 | 1.1779E-02 | 4.2506E-03 |
| 6 | 4.06 | 342.7 | 1.1778E-02 | 4.2506E-03 |
| 7 | 9.79 | 402.7 | 1.1778E-02 | 4.2491E-03 |
| 8 | 9.74 | 462.7 | 1.1779E-02 | 4.2492E-03 |
| 9 | 1.61 | 522.8 | 1.1777E-02 | 4.2501E-03 |
| 10 | 1.63 | 582.9 | 1.1776E-02 | 4.2501E-03 |

Mean: 1.1778E-02 4.2500E-03
Std Dev of fit (%): 0.10 0.15

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

Stable Isotope CF Analysis Results

File: DATA_008.raw
Project: controle2007.PRO
Sample list: 1704.spl
Line: 15
MS file: M-AN41
Inlet: GC-combustion
Inlet file: M-AN41
Sample ID:
Description: Calibration Mix Cal Acetate 001C

Acquisition Date: 31-7-2006 12:10:2
Weight: 0.00
Injection Volume: 10
Bottle:
Type:
Standard:
Slot Number: JB 251
Run Index:

| Reference standard | | | | | Corrections |
|-----------------------------------|--------|----------|--------|------|------------------------------|
| Species: CO2 by CF (uncalibrated) | | | | | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | | | | | |
| Ratio type: Elemental | | | | | |
| Deconvolution: Craig | | | | | |
| Elemental delta | | | | | |
| Molecular delta | | | | | |
| Label: | Value: | Label: | Value: | wrt: | |
| Ratio 1: 13C | -34.5 | delta 45 | -32.93 | PDB | |
| Ratio 2: 18O | -19.3 | delta 46 | -19.35 | PDB | |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 1.83 | 42.8 | 1.1777E-02 | 4.2513E-03 |
| 2 | 1.64 | 102.6 | 1.1776E-02 | 4.2514E-03 |
| 3 | 7.22 | 162.6 | 1.1779E-02 | 4.2502E-03 |
| 4 | 7.23 | 222.7 | 1.1779E-02 | 4.2502E-03 |
| 5 | 3.90 | 282.7 | 1.1778E-02 | 4.2516E-03 |
| 6 | 3.88 | 342.7 | 1.1778E-02 | 4.2510E-03 |
| 7 | 10.10 | 402.8 | 1.1778E-02 | 4.2493E-03 |
| 8 | 10.12 | 482.8 | 1.1778E-02 | 4.2496E-03 |
| 9 | 1.63 | 522.8 | 1.1776E-02 | 4.2522E-03 |
| 10 | 1.64 | 582.8 | 1.1776E-02 | 4.2516E-03 |

Mean: 1.1778E-02 4.2508E-03
Std Dev of fit (%): 0.09 0.24

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

Stable Isotope CF Analysis Results

| | |
|---|--------------------------------------|
| File: DATA_009.raw | Acquisition Date: 31-7-2006 12:21:28 |
| Project: controle2007.PRC | Weight: 0.00 |
| Sample list: 1704.spl | Injection Volume: 10 |
| Line: 15 | Bottle: |
| MS file: M-AN41 | Type: |
| Inlet: GC-combustion | Standard: |
| Inlet file: M-AN41 | Slot Number: JB 251 |
| Sample ID: | Run Index: |
| Description: Calibration Mix Cal Acetate 001C | |

| Reference standard | Corrections |
|-----------------------------------|-------------------------------|
| Species: CO2 by CF (uncalibrated) | Equilibrium correction: None |
| Gas: CO2 Uncalibrated CO2 | |
| Ratio type: Elemental | |
| Deconvolution: Craig | |
| Elemental delta | Molecular delta |
| Label: Value: | Label: Value: wrt: |
| Ratio 1: 13C -34.5 | delta 45 -32.93 PDB |
| Ratio 2: 18O -19.3 | delta 46 -19.35 PDB |

| Peak No | Major Height (nA) | RT (Sec) | Ratio 45/44 | Ratio 46/44 |
|---------|-------------------|----------|-------------|-------------|
| 1 | 1.64 | 42.7 | 1.1776E-02 | 4.2510E-03 |
| 2 | 1.64 | 102.7 | 1.1776E-02 | 4.2507E-03 |
| 3 | 7.41 | 162.6 | 1.1777E-02 | 4.2498E-03 |
| 4 | 7.43 | 222.7 | 1.1777E-02 | 4.2501E-03 |
| 5 | 3.83 | 282.7 | 1.1777E-02 | 4.2512E-03 |
| 6 | 3.83 | 342.7 | 1.1777E-02 | 4.2514E-03 |
| 7 | 9.96 | 402.7 | 1.1777E-02 | 4.2496E-03 |
| 8 | 9.98 | 462.8 | 1.1777E-02 | 4.2497E-03 |
| 9 | 1.61 | 522.9 | 1.1776E-02 | 4.2528E-03 |
| 10 | 1.62 | 582.9 | 1.1775E-02 | 4.2526E-03 |

Mean: 1.1777E-02 4.2509E-03
Std Dev of fit (%): 0.07 0.26

Sample Data

| Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta 13C | Ratio 46/44 | Raw Delta | delta 18O | delta 18O w.r.t. SMOW |
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|
|---------|----------|-------------------|------------|-------------|-----------|-----------|-------------|-----------|-----------|-----------------------|

180

Parameters: C:\MassLynx\lirms\CO2.prm

File | Reference Peak | **Sample Peak** | Beam | Peak Identification

Sample Peak Detection

Parameters

Window

Start (secs.) 0

End (secs.) 0

☒ Use full range

Baseline

☐ Peak Zero

Background Points

Offset (secs.) 3

Width (secs.) 1

☒ Ratio Polynomial

Polynomial

Order 2

Rejection 2

☐ Peak Start/End

☒ Use Horizontal

☐ Beam Zero

☐ Allow baseline to cut intensity

Save Help OK Cancel

COPIE CERTIFIÉE
CONFORME DES DOCUMENTS
ET FORMULAIRES ORIGINAUX

GDC01050

181

Parameters: C:\Masslynx\lirms\CO2.prm

File Reference Peak Sample Peak Beam Peak Identification

Peak Detection

Max. Flattop deviation (% height) 5

Peak Width (% FWHM) 60

Minimum Height (Å) 1000

Max. Group Separation (secs.) 300

Integration Limits

Fraction at Start 0.3

Fraction at End 0.1

Reference Ratio Polynomial

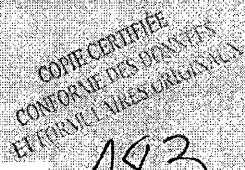
Order 0

Save Help OK Cancel

COTE CERTIFIEE
CONFORME DES SOCIETES
ET FORM L'UNES ORIGINEL

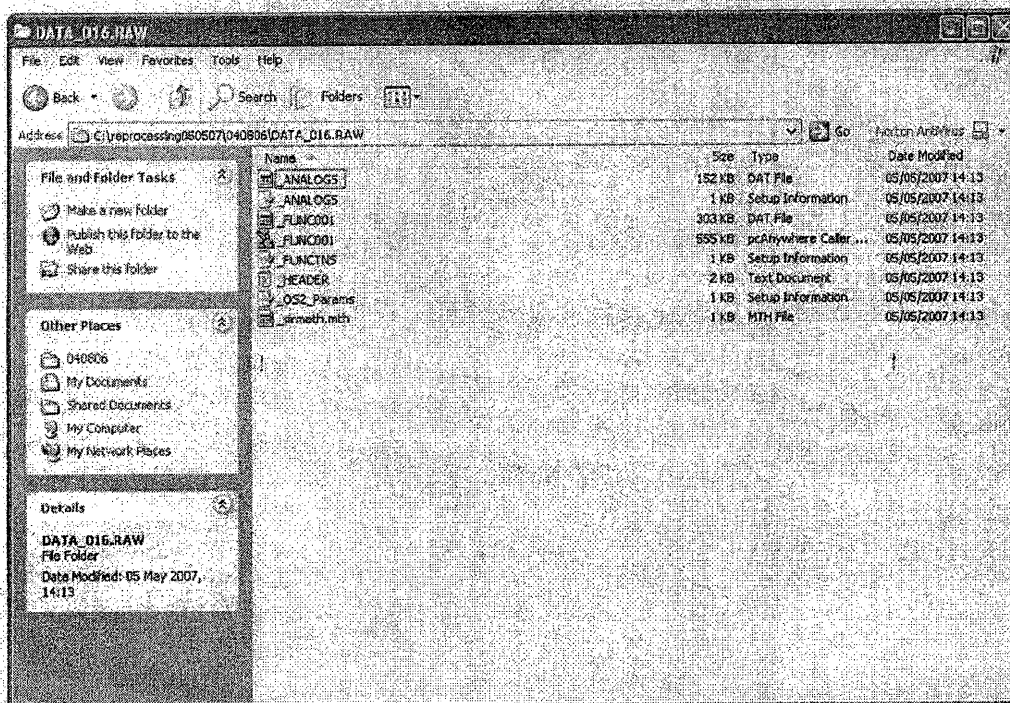
GDC01051

182





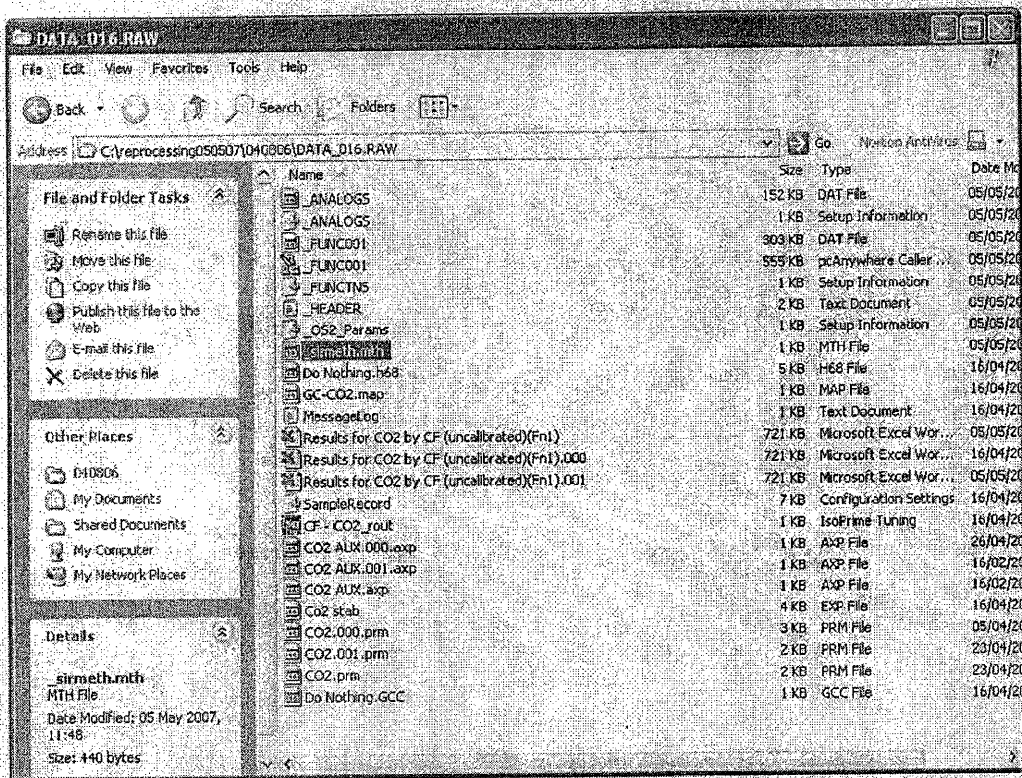
184



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

GDC01054

185



COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

GDC01055

186

17/04/2007 10:36:01 Starting inlet method - Kernel script
 17/04/2007 10:36:04 Opening Sample log file
 17/04/2007 10:36:15 Starting acquisition
 17/04/2007 10:40:18 Sample log file closed. Saved to:
 17/04/2007 10:40:18 C:\MassLynx Projects\controle2007.PRO\Data\1704bruit1.raw
 17/04/2007 10:40:18 Processing sample no 4 ...
 17/04/2007 10:40:19 ...processing complete
 17/04/2007 10:40:23 Kernel stopping inlet script
 17/04/2007 11:03:29 Starting inlet method - Kernel script
 17/04/2007 11:03:29 Opening Sample log file
 17/04/2007 11:03:40 Starting acquisition
 17/04/2007 11:13:51 Processing sample no 1 ...
 17/04/2007 11:13:52 Sample log file closed. Saved to:
 17/04/2007 11:13:52 C:\MassLynx Projects\controle2007.PRO\Data\1704stabilite1.raw
 17/04/2007 11:13:57 Kernel stopping inlet script
 17/04/2007 11:14:04 ...processing complete
 17/04/2007 11:14:07 Starting inlet method - Kernel script
 17/04/2007 11:14:08 Opening Sample log file
 17/04/2007 11:14:19 Starting acquisition
 17/04/2007 11:24:30 Sample log file closed. Saved to:
 17/04/2007 11:24:30 C:\MassLynx Projects\controle2007.PRO\Data\1704stabilite2.raw
 17/04/2007 11:24:31 Processing sample no 2 ...
 17/04/2007 11:24:33 Kernel stopping inlet script
 17/04/2007 11:24:45 ...processing complete
 17/04/2007 11:24:47 Starting inlet method - Kernel script
 17/04/2007 11:24:50 Opening Sample log file
 17/04/2007 11:25:01 Starting acquisition
 17/04/2007 11:35:12 Sample log file closed. Saved to:
 17/04/2007 11:35:12 C:\MassLynx Projects\controle2007.PRO\Data\1704stabilite3.raw
 17/04/2007 11:35:13 Processing sample no 3 ...
 17/04/2007 11:35:15 Kernel stopping inlet script
 17/04/2007 11:35:26 ...processing complete
 17/04/2007 11:42:48 Starting inlet method - Kernel script
 17/04/2007 11:42:51 Opening Sample log file
 17/04/2007 11:42:51 Commencing analysis of 1704MixCalIRMS01
 17/04/2007 11:42:51 Closing ref gas valves and waiting 30 sec
 17/04/2007 11:48:08 IsoPrime method stopped - WaitTillRunning
 17/04/2007 11:48:08 Sample log file closed. Saved to:
 17/04/2007 11:48:08 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalIRMS01.raw
 17/04/2007 11:48:11 Kernel stopping inlet script
 17/04/2007 11:49:41 Starting inlet method - Kernel script
 17/04/2007 11:49:41 Opening Sample log file
 17/04/2007 11:49:43 Commencing analysis of 1704MixCalIRMS01
 17/04/2007 11:49:43 Closing ref gas valves and waiting 30 sec
 17/04/2007 12:05:22 Sample log file closed. Saved to:
 17/04/2007 12:05:22 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalIRMS01.raw
 17/04/2007 12:05:22 Processing sample no 4 ...
 17/04/2007 12:05:25 Kernel stopping inlet script
 17/04/2007 12:05:38 ...processing complete
 17/04/2007 12:06:00 Starting inlet method - Kernel script
 17/04/2007 12:06:00 Opening Sample log file
 17/04/2007 12:06:00 Commencing analysis of 1704MixCalIRMS02
 17/04/2007 12:06:00 Closing ref gas valves and waiting 30 sec
 17/04/2007 12:16:25 Sample log file closed. Saved to:
 17/04/2007 12:16:25 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalIRMS02.raw
 17/04/2007 12:16:25 Kernel stopping inlet script
 17/04/2007 12:17:42 Starting inlet method - Kernel script
 17/04/2007 12:17:43 Opening Sample log file
 17/04/2007 12:17:43 Commencing analysis of 1704MixCalIRMS01
 17/04/2007 12:17:43 Closing ref gas valves and waiting 30 sec

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

187

GDC01056

17/04/2007 12:32:50 Sample log file closed. Saved to:
 17/04/2007 12:32:50 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalIRMS01.raw
 17/04/2007 12:32:50 Processing sample no 4 ...
 17/04/2007 12:32:53 Kernel stopping inlet script
 17/04/2007 12:33:05 ...processing complete
 17/04/2007 12:33:20 Starting inlet method - Kernel script
 17/04/2007 12:33:21 Opening Sample log file
 17/04/2007 12:33:21 Commencing analysis of 1704MixCalIRMS02
 17/04/2007 12:33:21 Closing ref gas valves and waiting 30 sec
 17/04/2007 12:48:27 Sample log file closed. Saved to:
 17/04/2007 12:48:27 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalIRMS02.raw
 17/04/2007 12:48:28 Processing sample no 5 ...
 17/04/2007 12:48:31 Kernel stopping inlet script
 17/04/2007 12:48:44 ...processing complete
 17/04/2007 12:49:03 Starting inlet method - Kernel script
 17/04/2007 12:49:06 Opening Sample log file
 17/04/2007 12:49:06 Commencing analysis of 1704MixCalIRMS03
 17/04/2007 12:49:06 Closing ref gas valves and waiting 30 sec
 17/04/2007 13:04:11 Processing sample no 6 ...
 17/04/2007 13:04:11 Sample log file closed. Saved to:
 17/04/2007 13:04:11 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalIRMS03.raw
 17/04/2007 13:04:15 Kernel stopping inlet script
 17/04/2007 13:04:23 ...processing complete
 17/04/2007 13:04:39 Starting inlet method - Kernel script
 17/04/2007 13:04:40 Opening Sample log file
 17/04/2007 13:04:40 Commencing analysis of 1704MixCalIRMS04
 17/04/2007 13:04:40 Closing ref gas valves and waiting 30 sec
 17/04/2007 13:19:46 Sample log file closed. Saved to:
 17/04/2007 13:19:46 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalIRMS04.raw
 17/04/2007 13:19:47 Processing sample no 7 ...
 17/04/2007 13:19:50 Kernel stopping inlet script
 17/04/2007 13:20:03 ...processing complete
 17/04/2007 13:20:57 Starting inlet method - Kernel script
 17/04/2007 13:21:00 Opening Sample log file
 17/04/2007 13:21:00 Commencing analysis of 1704MixCalAcetate01
 17/04/2007 13:21:00 Closing ref gas valves and waiting 30 sec
 17/04/2007 14:05:03 Sample log file closed. Saved to:
 17/04/2007 14:05:03 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalAcetate01.raw
 17/04/2007 14:05:04 Kernel stopping inlet script
 17/04/2007 14:05:04 Processing sample no 8 ...
 17/04/2007 14:05:20 ...processing complete
 17/04/2007 14:05:20 Starting inlet method - Kernel script
 17/04/2007 14:42:01 Opening Sample log file
 17/04/2007 14:42:01 Commencing analysis of 1704MixCalAcetate01
 17/04/2007 14:42:01 Closing ref gas valves and waiting 30 sec
 17/04/2007 15:26:12 Sample log file closed. Saved to:
 17/04/2007 15:26:12 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalAcetate01.raw
 17/04/2007 15:26:12 Processing sample no 8 ...
 17/04/2007 15:26:17 Kernel stopping inlet script
 17/04/2007 15:26:27 ...processing complete
 17/04/2007 15:32:49 Processing sample no 1 ...
 17/04/2007 15:33:01 ...processing complete
 17/04/2007 15:33:04 Processing sample no 2 ...
 17/04/2007 15:33:16 ...processing complete
 17/04/2007 15:33:18 Processing sample no 3 ...
 17/04/2007 15:33:30 ...processing complete
 17/04/2007 15:33:32 Processing sample no 4 ...
 17/04/2007 15:33:44 ...processing complete
 17/04/2007 15:33:46 Processing sample no 5 ...
 17/04/2007 15:33:56 ...processing complete

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

17/04/2007 15:33:58 Processing sample no 6 ...
 17/04/2007 15:34:10 ...processing complete
 17/04/2007 15:34:12 Processing sample no 7 ...
 17/04/2007 15:34:23 ...processing complete
 17/04/2007 15:34:25 Processing sample no 8 ...
 17/04/2007 15:34:38 ...processing complete
 17/04/2007 15:46:27 Processing sample no 1 ...
 17/04/2007 15:46:39 ...processing complete
 17/04/2007 15:46:42 Processing sample no 2 ...
 17/04/2007 15:46:54 ...processing complete
 17/04/2007 15:46:56 Processing sample no 3 ...
 17/04/2007 15:47:09 ...processing complete
 17/04/2007 15:47:12 Processing sample no 4 ...
 17/04/2007 15:47:24 ...processing complete
 17/04/2007 15:47:26 Processing sample no 5 ...
 17/04/2007 15:47:38 ...processing complete
 17/04/2007 15:47:41 Processing sample no 6 ...
 17/04/2007 15:47:52 ...processing complete
 17/04/2007 15:47:54 Processing sample no 7 ...
 17/04/2007 15:48:09 ...processing complete
 17/04/2007 15:48:12 Processing sample no 8 ...
 17/04/2007 15:48:26 ...processing complete
 17/04/2007 18:21:42 Starting inlet method - Kernel script
 17/04/2007 18:21:42 Opening Sample log file
 17/04/2007 18:21:42 Commencing analysis of 1704Blu1F3
 17/04/2007 18:21:42 Closing ref gas valves and waiting 30 sec
 17/04/2007 19:05:53 Sample log file closed. Saved to:
 17/04/2007 19:05:53 C:\MassLynx Projects\controle2007.PRO\Data\1704Blu1F3.raw
 17/04/2007 19:05:54 Processing sample no 9 ...
 17/04/2007 19:05:54 Kernel stopping inlet script
 17/04/2007 19:06:10 ...processing complete
 17/04/2007 19:06:24 Starting inlet method - Kernel script
 17/04/2007 19:06:24 Opening Sample log file
 17/04/2007 19:06:24 Commencing analysis of 1704429F3
 17/04/2007 19:06:24 Closing ref gas valves and waiting 30 sec
 17/04/2007 19:50:31 Processing sample no 10 ...
 17/04/2007 19:50:31 Sample log file closed. Saved to:
 17/04/2007 19:50:31 C:\MassLynx Projects\controle2007.PRO\Data\1704429F3.raw
 17/04/2007 19:50:36 Kernel stopping inlet script
 17/04/2007 19:50:45 ...processing complete
 17/04/2007 19:52:42 Starting inlet method - Kernel script
 17/04/2007 19:52:42 Opening Sample log file
 17/04/2007 19:52:43 Commencing analysis of 1704Blu1F2
 17/04/2007 19:52:43 Closing ref gas valves and waiting 30 sec
 17/04/2007 20:17:24 Sample log file closed. Saved to:
 17/04/2007 20:17:24 C:\MassLynx Projects\controle2007.PRO\Data\1704Blu1F2.raw
 17/04/2007 20:17:25 Kernel stopping inlet script
 17/04/2007 20:24:28 Starting inlet method - Kernel script
 17/04/2007 20:24:29 Opening Sample log file
 17/04/2007 20:24:29 Commencing analysis of 1704Blu1F2
 17/04/2007 20:24:29 Closing ref gas valves and waiting 30 sec
 17/04/2007 21:08:36 Sample log file closed. Saved to:
 17/04/2007 21:08:36 C:\MassLynx Projects\controle2007.PRO\Data\1704Blu1F2.raw
 17/04/2007 21:08:37 Processing sample no 11 ...
 17/04/2007 21:08:41 Kernel stopping inlet script
 17/04/2007 21:08:51 ...processing complete
 17/04/2007 21:09:06 Starting inlet method - Kernel script
 17/04/2007 21:09:09 Opening Sample log file
 17/04/2007 21:09:09 Commencing analysis of 1704429F2
 17/04/2007 21:09:09 Closing ref gas valves and waiting 30 sec

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

17/04/2007 21:53:20 Sample log file closed. Saved to:
 17/04/2007 21:53:20 C:\MassLynx Projects\controle2007.PRO\Data\1704429F2.raw
 17/04/2007 21:53:21 Processing sample no 12 ...
 17/04/2007 21:53:24 Kernel stopping inlet script
 17/04/2007 21:53:39 ...processing complete
 17/04/2007 21:53:55 Starting inlet method - Kernel script
 17/04/2007 21:53:56 Opening Sample log file
 17/04/2007 21:53:56 Commencing analysis of 1704Blu1F1
 17/04/2007 21:53:56 Closing ref gas valves and waiting 30 sec
 17/04/2007 22:38:01 Sample log file closed. Saved to:
 17/04/2007 22:38:01 C:\MassLynx Projects\controle2007.PRO\Data\1704Blu1F1.raw
 17/04/2007 22:38:02 Processing sample no 13 ...
 17/04/2007 22:38:03 Kernel stopping inlet script
 17/04/2007 22:38:17 ...processing complete
 17/04/2007 22:38:31 Starting inlet method - Kernel script
 17/04/2007 22:38:32 Opening Sample log file
 17/04/2007 22:38:32 Commencing analysis of 1704429F1
 17/04/2007 22:38:32 Closing ref gas valves and waiting 30 sec
 17/04/2007 23:22:45 Sample log file closed. Saved to:
 17/04/2007 23:22:45 C:\MassLynx Projects\controle2007.PRO\Data\1704429F1.raw
 17/04/2007 23:22:45 Kernel stopping inlet script
 17/04/2007 23:22:45 Processing sample no 14 ...
 17/04/2007 23:23:01 ...processing complete
 17/04/2007 23:23:18 Starting inlet method - Kernel script
 17/04/2007 23:23:18 Opening Sample log file
 17/04/2007 23:23:19 Commencing analysis of 1704MixCalAcetate02
 17/04/2007 23:23:19 Closing ref gas valves and waiting 30 sec
 18/04/2007 00:07:24 Sample log file closed. Saved to:
 18/04/2007 00:07:24 C:\MassLynx Projects\controle2007.PRO\Data\1704MixCalAcetate02.raw
 18/04/2007 00:07:25 Kernel stopping inlet script
 18/04/2007 00:07:25 Processing sample no 15 ...
 18/04/2007 00:07:40 ...processing complete
 18/04/2007 00:07:54 Starting inlet method - Kernel script
 18/04/2007 00:07:57 Opening Sample log file
 18/04/2007 00:07:57 Commencing analysis of 1704Hexane
 18/04/2007 00:07:57 Closing ref gas valves and waiting 30 sec
 18/04/2007 00:52:07 Sample log file closed. Saved to:
 18/04/2007 00:52:07 C:\MassLynx Projects\controle2007.PRO\Data\1704Hexane.raw
 18/04/2007 00:52:08 Processing sample no 16 ...
 18/04/2007 00:52:08 Kernel stopping inlet script
 18/04/2007 00:52:23 ...processing complete
 18/04/2007 09:07:39 Opening Sample log file
 18/04/2007 09:07:49 Starting acquisition
 18/04/2007 09:18:01 Sample log file closed. Saved to:
 18/04/2007 09:18:01 C:\MassLynx Projects\controle2007.PRO\Data\1804stabilite1.raw
 18/04/2007 09:18:01 Processing sample no 1 ...
 18/04/2007 09:18:04 Kernel stopping inlet script
 18/04/2007 09:18:15 ...processing complete
 18/04/2007 09:18:17 Starting inlet method - Kernel script
 18/04/2007 09:18:18 Opening Sample log file
 18/04/2007 09:18:29 Starting acquisition
 18/04/2007 09:28:40 Sample log file closed. Saved to:
 18/04/2007 09:28:40 C:\MassLynx Projects\controle2007.PRO\Data\1804stabilite2.raw
 18/04/2007 09:28:41 Processing sample no 2 ...
 18/04/2007 09:28:45 Kernel stopping inlet script
 18/04/2007 09:28:54 ...processing complete
 18/04/2007 09:28:57 Starting inlet method - Kernel script
 18/04/2007 09:29:02 Opening Sample log file
 18/04/2007 09:29:12 Starting acquisition

COPIE CERTIFIÉE
 CONFORME DES ORIGINAUX
 ET FORMULAIRES ORIGINAUX

18/04/2007 09:39:24 Sample log file closed. Saved to:
18/04/2007 09:39:24 C:\MassLynx Projects\controle2007.PRO\Data\1804stabilite3.raw
18/04/2007 09:39:25 Processing sample no 3 ...
18/04/2007 09:39:27 Kernel stopping inlet script
18/04/2007 09:39:37 ...processing complete
18/04/2007 09:49:40 Starting inlet method - Kernel script
18/04/2007 09:49:41 Opening Sample log file
18/04/2007 09:49:41 Commencing analysis of 1804MixCalIRMS01
18/04/2007 09:49:41 Closing ref gas valves and waiting 30 sec
18/04/2007 10:04:53 Sample log file closed. Saved to:
18/04/2007 10:04:53 C:\MassLynx Projects\controle2007.PRO\Data\1804MixCalIRMS01.raw
18/04/2007 10:04:54 Processing sample no 4 ...
18/04/2007 10:04:57 Kernel stopping inlet script
18/04/2007 10:05:11 ...processing complete
18/04/2007 10:05:27 Starting inlet method - Kernel script
18/04/2007 10:05:27 Opening Sample log file
18/04/2007 10:05:27 Commencing analysis of 1804MixCalIRMS02
18/04/2007 10:05:28 Closing ref gas valves and waiting 30 sec
18/04/2007 10:20:34 Sample log file closed. Saved to:
18/04/2007 10:20:34 C:\MassLynx Projects\controle2007.PRO\Data\1804MixCalIRMS02.raw
18/04/2007 10:20:35 Processing sample no 5 ...
18/04/2007 10:20:37 Kernel stopping inlet script
18/04/2007 10:20:48 ...processing complete
18/04/2007 10:21:02 Starting inlet method - Kernel script
18/04/2007 10:21:05 Opening Sample log file
18/04/2007 10:21:05 Commencing analysis of 1804MixCalIRMS03
18/04/2007 10:21:05 Closing ref gas valves and waiting 30 sec
18/04/2007 10:36:08 Sample log file closed. Saved to:
18/04/2007 10:36:08 C:\MassLynx Projects\controle2007.PRO\Data\1804MixCalIRMS03.raw
18/04/2007 10:36:09 Processing sample no 6 ...
18/04/2007 10:36:12 Kernel stopping inlet script
18/04/2007 10:36:23 ...processing complete
18/04/2007 10:37:18 Starting inlet method - Kernel script
18/04/2007 10:37:21 Opening Sample log file
18/04/2007 10:37:21 Commencing analysis of 1804MixCalAcetate01
18/04/2007 10:37:21 Closing ref gas valves and waiting 30 sec
18/04/2007 11:21:24 Sample log file closed. Saved to:
18/04/2007 11:21:24 C:\MassLynx Projects\controle2007.PRO\Data\1804MixCalAcetate01.raw
18/04/2007 11:21:25 Kernel stopping inlet script
18/04/2007 11:21:25 Processing sample no 7 ...
18/04/2007 11:21:41 ...processing complete
18/04/2007 12:16:27 Opening Sample log file
18/04/2007 12:16:27 Commencing analysis of 1804Blu1F3
18/04/2007 12:16:27 Closing ref gas valves and waiting 30 sec
18/04/2007 13:00:40 Sample log file closed. Saved to:
18/04/2007 13:00:40 C:\MassLynx Projects\controle2007.PRO\Data\1804Blu1F3.raw
18/04/2007 13:00:41 Processing sample no 8 ...
18/04/2007 13:00:45 Kernel stopping inlet script
18/04/2007 13:00:55 ...processing complete
18/04/2007 13:01:09 Starting inlet method - Kernel script
18/04/2007 13:01:12 Opening Sample log file
18/04/2007 13:01:12 Commencing analysis of 1804855F3
18/04/2007 13:01:12 Closing ref gas valves and waiting 30 sec
18/04/2007 13:45:16 Sample log file closed. Saved to:
18/04/2007 13:45:16 C:\MassLynx Projects\controle2007.PRO\Data\1804855F3.raw
18/04/2007 13:45:16 Kernel stopping inlet script
18/04/2007 13:45:16 Processing sample no 9 ...
18/04/2007 13:45:31 ...processing complete
18/04/2007 13:54:35 Starting inlet method - Kernel script

COPIED
CONFORMED
ET FORVILLES

GDC01060

18/04/2007 13:54:36 Opening Sample log file
 18/04/2007 13:54:36 Commencing analysis of 1804855F3-2
 18/04/2007 13:54:36 Closing ref gas valves and waiting 30 sec
 18/04/2007 14:38:48 Sample log file closed. Saved to:
 18/04/2007 14:38:48 C:\MassLynx Projects\controle2007.PRO\Data\1804855F3-2.raw
 18/04/2007 14:38:48 Processing sample no 10 ...
 18/04/2007 14:38:52 Kernel stopping inlet script
 18/04/2007 14:39:03 ...processing complete
 18/04/2007 14:39:22 Starting inlet method - Kernel script
 18/04/2007 14:39:25 Opening Sample log file
 18/04/2007 14:39:25 Commencing analysis of 1804Blu1F2
 18/04/2007 14:39:25 Closing ref gas valves and waiting 30 sec
 18/04/2007 15:23:29 Sample log file closed. Saved to:
 18/04/2007 15:23:29 C:\MassLynx Projects\controle2007.PRO\Data\1804Blu1F2.raw
 18/04/2007 15:23:30 Kernel stopping inlet script
 18/04/2007 15:23:30 Processing sample no 11 ...
 18/04/2007 15:23:45 ...processing complete
 18/04/2007 15:24:02 Starting inlet method - Kernel script
 18/04/2007 15:24:03 Opening Sample log file
 18/04/2007 15:24:03 Commencing analysis of 1804855F2
 18/04/2007 15:24:03 Closing ref gas valves and waiting 30 sec
 18/04/2007 16:08:16 Sample log file closed. Saved to:
 18/04/2007 16:08:16 C:\MassLynx Projects\controle2007.PRO\Data\1804855F2.raw
 18/04/2007 16:08:16 Processing sample no 12 ...
 18/04/2007 16:08:19 Kernel stopping inlet script
 18/04/2007 16:08:33 ...processing complete
 18/04/2007 16:08:47 Starting inlet method - Kernel script
 18/04/2007 16:08:48 Opening Sample log file
 18/04/2007 16:08:48 Commencing analysis of 1804Blu1F1
 18/04/2007 16:08:48 Closing ref gas valves and waiting 30 sec
 18/04/2007 16:52:54 Sample log file closed. Saved to:
 18/04/2007 16:52:54 C:\MassLynx Projects\controle2007.PRO\Data\1804Blu1F1.raw
 18/04/2007 16:52:54 Kernel stopping inlet script
 18/04/2007 16:52:55 Processing sample no 13 ...
 18/04/2007 16:53:08 ...processing complete
 18/04/2007 16:53:22 Starting inlet method - Kernel script
 18/04/2007 16:53:25 Opening Sample log file
 18/04/2007 16:53:25 Commencing analysis of 1804855F1
 18/04/2007 16:53:25 Closing ref gas valves and waiting 30 sec
 18/04/2007 17:37:35 Sample log file closed. Saved to:
 18/04/2007 17:37:35 C:\MassLynx Projects\controle2007.PRO\Data\1804855F1.raw
 18/04/2007 17:37:35 Processing sample no 14 ...
 18/04/2007 17:37:40 Kernel stopping inlet script
 18/04/2007 17:37:52 ...processing complete
 18/04/2007 18:28:27 Starting inlet method - Kernel script
 18/04/2007 18:28:28 Opening Sample log file
 18/04/2007 18:28:28 Commencing analysis of 1804Blu2F3
 18/04/2007 18:28:28 Closing ref gas valves and waiting 30 sec
 18/04/2007 19:12:30 Sample log file closed. Saved to:
 18/04/2007 19:12:30 C:\MassLynx Projects\controle2007.PRO\Data\1804Blu2F3.raw
 18/04/2007 19:12:31 Processing sample no 15 ...
 18/04/2007 19:12:34 Kernel stopping inlet script
 18/04/2007 19:12:45 ...processing complete
 18/04/2007 19:29:17 Starting inlet method - Kernel script
 18/04/2007 19:29:20 Opening Sample log file
 18/04/2007 19:29:20 Commencing analysis of 1804423F3
 18/04/2007 19:29:20 Closing ref gas valves and waiting 30 sec
 18/04/2007 20:10:05 Sample log file closed. Saved to:
 18/04/2007 20:10:05 C:\MassLynx Projects\controle2007.PRO\Data\1804423F3.raw
 18/04/2007 20:10:05 Processing sample no 16 ...

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

GDC01061

107

18/04/2007 20:10:09 Kernel stopping inlet script
18/04/2007 20:10:20 ...processing complete
18/04/2007 20:10:42 Starting inlet method - Kernel script
18/04/2007 20:10:43 Opening Sample log file
18/04/2007 20:10:43 Commencing analysis of 1804Blu2F2
18/04/2007 20:10:43 Closing ref gas valves and waiting 30 sec
18/04/2007 20:54:50 Sample log file closed. Saved to:
18/04/2007 20:54:50 C:\MassLynx Projects\controle2007.PRO\Data\1804Blu2F2.raw
18/04/2007 20:54:51 Processing sample no 17 ...
18/04/2007 20:54:55 Kernel stopping inlet script
18/04/2007 20:55:14 ...processing complete
18/04/2007 20:55:29 Starting inlet method - Kernel script
18/04/2007 20:55:30 Opening Sample log file
18/04/2007 20:55:30 Commencing analysis of 1804423F2
18/04/2007 20:55:30 Closing ref gas valves and waiting 30 sec
18/04/2007 21:39:42 Sample log file closed. Saved to:
18/04/2007 21:39:42 C:\MassLynx Projects\controle2007.PRO\Data\1804423F2.raw
18/04/2007 21:39:42 Processing sample no 18 ...
18/04/2007 21:39:47 Kernel stopping inlet script
18/04/2007 21:39:56 ...processing complete
18/04/2007 21:40:10 Starting inlet method - Kernel script
18/04/2007 21:40:11 Opening Sample log file
18/04/2007 21:40:11 Commencing analysis of 1804Blu2F1
18/04/2007 21:40:11 Closing ref gas valves and waiting 30 sec
18/04/2007 22:24:16 Sample log file closed. Saved to:
18/04/2007 22:24:16 C:\MassLynx Projects\controle2007.PRO\Data\1804Blu2F1.raw
18/04/2007 22:24:17 Processing sample no 19 ...
18/04/2007 22:24:22 Kernel stopping inlet script
18/04/2007 22:24:32 ...processing complete
18/04/2007 22:24:46 Starting inlet method - Kernel script
18/04/2007 22:24:47 Opening Sample log file
18/04/2007 22:24:47 Commencing analysis of 1804423F1
18/04/2007 22:24:47 Closing ref gas valves and waiting 30 sec
18/04/2007 23:08:59 Sample log file closed. Saved to:
18/04/2007 23:08:59 C:\MassLynx Projects\controle2007.PRO\Data\1804423F1.raw
18/04/2007 23:08:59 Kernel stopping inlet script
18/04/2007 23:09:00 Processing sample no 20 ...
18/04/2007 23:09:15 ...processing complete
18/04/2007 23:09:29 Starting inlet method - Kernel script
18/04/2007 23:09:32 Opening Sample log file
18/04/2007 23:09:32 Commencing analysis of 1804MixCalAcetate02
18/04/2007 23:09:32 Closing ref gas valves and waiting 30 sec
18/04/2007 23:53:35 Sample log file closed. Saved to:
18/04/2007 23:53:35 C:\MassLynx Projects\controle2007.PRO\Data\1804MixCalAcetate02.raw
18/04/2007 23:53:35 Kernel stopping inlet script
18/04/2007 23:53:36 Processing sample no 21 ...
18/04/2007 23:53:50 ...processing complete
19/04/2007 09:32:07 Starting inlet method - Kernel script
19/04/2007 09:32:08 Opening Sample log file
19/04/2007 09:32:18 Starting acquisition
19/04/2007 09:42:29 Processing sample no 1 ...
19/04/2007 09:42:30 Sample log file closed. Saved to:
19/04/2007 09:42:30 C:\MassLynx Projects\controle2007.PRO\Data\1904stabilite1.raw
19/04/2007 09:42:33 Kernel stopping inlet script
19/04/2007 09:42:40 ...processing complete
19/04/2007 09:42:44 Starting inlet method - Kernel script
19/04/2007 09:42:44 Opening Sample log file
19/04/2007 09:42:55 Starting acquisition
19/04/2007 09:53:06 Processing sample no 2 ...
19/04/2007 09:53:06 Sample log file closed. Saved to:

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

197

19/04/2007 09:53:06 C:\MassLynx Projects\controle2007.PRO\Data\1904stabilite2.raw
19/04/2007 09:53:09 Kernel stopping inlet script
19/04/2007 09:53:19 ...processing complete
19/04/2007 09:53:22 Starting inlet method - Kernel script
19/04/2007 09:53:25 Opening Sample log file
19/04/2007 09:53:36 Starting acquisition
19/04/2007 10:03:48 Sample log file closed. Saved to:
19/04/2007 10:03:48 C:\MassLynx Projects\controle2007.PRO\Data\1904stabilite3.raw
19/04/2007 10:03:48 Processing sample no 3 ...
19/04/2007 10:03:51 Kernel stopping inlet script
19/04/2007 10:04:00 ...processing complete
19/04/2007 10:05:39 Starting inlet method - Kernel script
19/04/2007 10:05:40 Opening Sample log file
19/04/2007 10:05:40 Commencing analysis of 1904MixCalIRMS01
19/04/2007 10:05:40 Closing ref gas valves and waiting 30 sec
19/04/2007 10:20:52 Sample log file closed. Saved to:
19/04/2007 10:20:52 C:\MassLynx Projects\controle2007.PRO\Data\1904MixCalIRMS01.raw
19/04/2007 10:20:53 Processing sample no 4 ...
19/04/2007 10:20:53 Kernel stopping inlet script
19/04/2007 10:21:07 ...processing complete
19/04/2007 10:21:20 Starting inlet method - Kernel script
19/04/2007 10:21:21 Opening Sample log file
19/04/2007 10:21:21 Commencing analysis of 1904MixCalIRMS02
19/04/2007 10:21:21 Closing ref gas valves and waiting 30 sec
19/04/2007 10:36:24 Sample log file closed. Saved to:
19/04/2007 10:36:24 C:\MassLynx Projects\controle2007.PRO\Data\1904MixCalIRMS02.raw
19/04/2007 10:36:24 Processing sample no 5 ...
19/04/2007 10:36:25 Kernel stopping inlet script
19/04/2007 10:36:38 ...processing complete
19/04/2007 10:36:57 Starting inlet method - Kernel script
19/04/2007 10:37:00 Opening Sample log file
19/04/2007 10:37:00 Commencing analysis of 1904MixCalIRMS03
19/04/2007 10:37:00 Closing ref gas valves and waiting 30 sec
19/04/2007 10:52:04 Sample log file closed. Saved to:
19/04/2007 10:52:04 C:\MassLynx Projects\controle2007.PRO\Data\1904MixCalIRMS03.raw
19/04/2007 10:52:05 Processing sample no 6 ...
19/04/2007 10:52:07 Kernel stopping inlet script
19/04/2007 10:52:15 ...processing complete
19/04/2007 11:09:42 Starting inlet method - Kernel script
19/04/2007 11:09:45 Opening Sample log file
19/04/2007 11:09:45 Commencing analysis of 1904MixCalAcetate01
19/04/2007 11:09:45 Closing ref gas valves and waiting 30 sec
19/04/2007 11:53:55 Sample log file closed. Saved to:
19/04/2007 11:53:55 C:\MassLynx Projects\controle2007.PRO\Data\1904MixCalAcetate01.raw
19/04/2007 11:53:55 Kernel stopping inlet script
19/04/2007 11:53:55 Processing sample no 7 ...
19/04/2007 11:54:09 ...processing complete
19/04/2007 12:29:52 Starting inlet method - Kernel script
19/04/2007 12:29:55 Opening Sample log file
19/04/2007 12:29:55 Commencing analysis of 1904Blu1F3
19/04/2007 12:29:56 Closing ref gas valves and waiting 30 sec
19/04/2007 13:14:06 Sample log file closed. Saved to:
19/04/2007 13:14:06 C:\MassLynx Projects\controle2007.PRO\Data\1904Blu1F3.raw
19/04/2007 13:14:07 Processing sample no 8 ...
19/04/2007 13:14:12 Kernel stopping inlet script
19/04/2007 13:14:21 ...processing complete
19/04/2007 13:16:04 Starting inlet method - Kernel script
19/04/2007 13:16:07 Opening Sample log file
19/04/2007 13:16:07 Commencing analysis of 1904426F3
19/04/2007 13:16:07 Closing ref gas valves and waiting 30 sec

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINALS

19.

19/04/2007 14:00:11 Sample log file closed. Saved to:
19/04/2007 14:00:11 C:\MassLynx Projects\controle2007.PRO\Data\1904426F3.raw
19/04/2007 14:00:11 Kernel stopping inlet script
19/04/2007 14:00:11 Processing sample no 9 ...
19/04/2007 14:00:14 RefLibAPI.ReadData: Operation is not allowed when the object is closed.
19/04/2007 14:00:14 Unable to read CO2 by CF (uncalibrated) data from reference library
19/04/2007 14:00:15 ...processing complete
19/04/2007 14:02:04 Starting inlet method - Kernel script
19/04/2007 14:02:05 Opening Sample log file
19/04/2007 14:02:05 Commencing analysis of 1904Blu1F2
19/04/2007 14:02:05 Closing ref gas valves and waiting 30 sec
19/04/2007 14:46:20 Sample log file closed. Saved to:
19/04/2007 14:46:20 C:\MassLynx Projects\controle2007.PRO\Data\1904Blu1F2.raw
19/04/2007 14:46:20 Kernel stopping inlet script
19/04/2007 14:46:20 Processing sample no 10 ...
19/04/2007 14:46:32 ...processing complete
19/04/2007 14:46:52 Starting inlet method - Kernel script
19/04/2007 14:46:55 Opening Sample log file
19/04/2007 14:46:55 Commencing analysis of 1904426F2
19/04/2007 14:46:55 Closing ref gas valves and waiting 30 sec
19/04/2007 15:30:59 Sample log file closed. Saved to:
19/04/2007 15:30:59 C:\MassLynx Projects\controle2007.PRO\Data\1904426F2.raw
19/04/2007 15:31:00 Processing sample no 11 ...
19/04/2007 15:31:04 Kernel stopping inlet script
19/04/2007 15:31:14 ...processing complete
19/04/2007 15:39:16 Starting inlet method - Kernel script
19/04/2007 15:39:17 Opening Sample log file
19/04/2007 15:39:17 Commencing analysis of 1904Blu1F1
19/04/2007 15:39:17 Closing ref gas valves and waiting 30 sec
19/04/2007 16:23:30 Sample log file closed. Saved to:
19/04/2007 16:23:30 C:\MassLynx Projects\controle2007.PRO\Data\1904Blu1F1.raw
19/04/2007 16:23:31 Processing sample no 12 ...
19/04/2007 16:23:35 Kernel stopping inlet script
19/04/2007 16:23:46 ...processing complete
19/04/2007 16:24:00 Starting inlet method - Kernel script
19/04/2007 16:24:03 Opening Sample log file
19/04/2007 16:24:03 Commencing analysis of 1904426F1
19/04/2007 16:24:03 Closing ref gas valves and waiting 30 sec
19/04/2007 17:08:06 Sample log file closed. Saved to:
19/04/2007 17:08:06 C:\MassLynx Projects\controle2007.PRO\Data\1904426F1.raw
19/04/2007 17:08:07 Processing sample no 13 ...
19/04/2007 17:08:07 Kernel stopping inlet script
19/04/2007 17:08:22 ...processing complete
19/04/2007 18:03:46 Starting inlet method - Kernel script
19/04/2007 18:03:47 Opening Sample log file
19/04/2007 18:03:47 Commencing analysis of 1904Blu2F3
19/04/2007 18:03:47 Closing ref gas valves and waiting 30 sec
19/04/2007 18:48:00 Sample log file closed. Saved to:
19/04/2007 18:48:00 C:\MassLynx Projects\controle2007.PRO\Data\1904Blu2F3.raw
19/04/2007 18:48:01 Processing sample no 14 ...
19/04/2007 18:48:05 Kernel stopping inlet script
19/04/2007 18:48:14 ...processing complete
19/04/2007 18:48:28 Starting inlet method - Kernel script
19/04/2007 18:48:31 Opening Sample log file
19/04/2007 18:48:31 Commencing analysis of 1904428F3
19/04/2007 18:48:31 Closing ref gas valves and waiting 30 sec
19/04/2007 19:32:36 Sample log file closed. Saved to:
19/04/2007 19:32:36 C:\MassLynx Projects\controle2007.PRO\Data\1904428F3.raw
19/04/2007 19:32:37 Processing sample no 15 ...
19/04/2007 19:32:41 Kernel stopping inlet script

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

19/04/2007 19:32:52 ...processing complete
 19/04/2007 19:33:08 Starting inlet method - Kernel script
 19/04/2007 19:33:11 Opening Sample log file
 19/04/2007 19:33:11 Commencing analysis of 1904428F3-2
 19/04/2007 19:33:11 Closing ref gas valves and waiting 30 sec
 19/04/2007 20:17:22 Sample log file closed. Saved to:
 19/04/2007 20:17:22 C:\MassLynx Projects\controle2007.PRO\Data\1904428F3-2.raw
 19/04/2007 20:17:22 Processing sample no 16 ...
 19/04/2007 20:17:22 Kernel stopping inlet script
 19/04/2007 20:17:37 ...processing complete
 19/04/2007 20:17:53 Starting inlet method - Kernel script
 19/04/2007 20:17:54 Opening Sample log file
 19/04/2007 20:17:54 Commencing analysis of 1904Blu2F2
 19/04/2007 20:17:54 Closing ref gas valves and waiting 30 sec
 19/04/2007 21:01:59 Sample log file closed. Saved to:
 19/04/2007 21:01:59 C:\MassLynx Projects\controle2007.PRO\Data\1904Blu2F2.raw
 19/04/2007 21:02:00 Processing sample no 17 ...
 19/04/2007 21:02:05 Kernel stopping inlet script
 19/04/2007 21:02:13 ...processing complete
 19/04/2007 21:02:27 Starting inlet method - Kernel script
 19/04/2007 21:02:28 Opening Sample log file
 19/04/2007 21:02:28 Commencing analysis of 1904428F2
 19/04/2007 21:02:28 Closing ref gas valves and waiting 30 sec
 19/04/2007 21:46:40 Sample log file closed. Saved to:
 19/04/2007 21:46:40 C:\MassLynx Projects\controle2007.PRO\Data\1904428F2.raw
 19/04/2007 21:46:41 Processing sample no 18 ...
 19/04/2007 21:46:46 Kernel stopping inlet script
 19/04/2007 21:46:56 ...processing complete
 19/04/2007 21:47:11 Starting inlet method - Kernel script
 19/04/2007 21:47:11 Opening Sample log file
 19/04/2007 21:47:11 Commencing analysis of 1904Blu2F1
 19/04/2007 21:47:12 Closing ref gas valves and waiting 30 sec
 19/04/2007 22:31:18 Processing sample no 19 ...
 19/04/2007 22:31:18 Sample log file closed. Saved to:
 19/04/2007 22:31:18 C:\MassLynx Projects\controle2007.PRO\Data\1904Blu2F1.raw
 19/04/2007 22:31:23 Kernel stopping inlet script
 19/04/2007 22:31:38 ...processing complete
 19/04/2007 22:31:52 Starting inlet method - Kernel script
 19/04/2007 22:31:53 Opening Sample log file
 19/04/2007 22:31:53 Commencing analysis of 1904428F1
 19/04/2007 22:31:53 Closing ref gas valves and waiting 30 sec
 19/04/2007 23:16:06 Sample log file closed. Saved to:
 19/04/2007 23:16:06 C:\MassLynx Projects\controle2007.PRO\Data\1904428F1.raw
 19/04/2007 23:16:06 Processing sample no 20 ...
 19/04/2007 23:16:11 Kernel stopping inlet script
 19/04/2007 23:16:19 ...processing complete
 19/04/2007 23:16:33 Starting inlet method - Kernel script
 19/04/2007 23:16:36 Opening Sample log file
 19/04/2007 23:16:36 Commencing analysis of 1904MixCalAcetate02
 19/04/2007 23:16:36 Closing ref gas valves and waiting 30 sec
 20/04/2007 00:00:40 Sample log file closed. Saved to:
 20/04/2007 00:00:40 C:\MassLynx Projects\controle2007.PRO\Data\1904MixCalAcetate02.raw
 20/04/2007 00:00:40 Processing sample no 21 ...
 20/04/2007 00:00:45 Kernel stopping inlet script
 20/04/2007 00:00:54 ...processing complete
 20/04/2007 09:38:47 Starting inlet method - Kernel script
 20/04/2007 09:38:48 Opening Sample log file
 20/04/2007 09:38:58 Starting acquisition
 20/04/2007 09:49:10 Sample log file closed. Saved to:
 20/04/2007 09:49:10 C:\MassLynx Projects\controle2007.PRO\Data\2004stabilite1.raw

COPIE CERTIFIÉE
 CONFORME DES PROCÉDÉS
 ET FORMULAIRES ORIGINELS

20/04/2007 09:49:12 Processing sample no 1 ...
 20/04/2007 09:49:15 Kernel stopping inlet script
 20/04/2007 09:49:25 ...processing complete
 20/04/2007 09:49:30 Starting inlet method - Kernel script
 20/04/2007 09:49:31 Opening Sample log file
 20/04/2007 09:49:41 Starting acquisition
 20/04/2007 09:59:53 Sample log file closed. Saved to:
 20/04/2007 09:59:53 C:\MassLynx Projects\controle2007.PRO\Data\2004stabilite2.raw
 20/04/2007 09:59:54 Processing sample no 2 ...
 20/04/2007 09:59:56 Kernel stopping inlet script
 20/04/2007 10:00:06 ...processing complete
 20/04/2007 10:00:09 Starting inlet method - Kernel script
 20/04/2007 10:00:10 Opening Sample log file
 20/04/2007 10:00:20 Starting acquisition
 20/04/2007 10:10:32 Sample log file closed. Saved to:
 20/04/2007 10:10:32 C:\MassLynx Projects\controle2007.PRO\Data\2004stabilite3.raw
 20/04/2007 10:10:32 Processing sample no 3 ...
 20/04/2007 10:10:32 Kernel stopping inlet script
 20/04/2007 10:10:45 ...processing complete
 20/04/2007 10:12:17 Starting inlet method - Kernel script
 20/04/2007 10:12:20 Opening Sample log file
 20/04/2007 10:12:20 Commencing analysis of 2004MixCalIRMS01
 20/04/2007 10:12:20 Closing ref gas valves and waiting 30 sec
 20/04/2007 10:27:30 Sample log file closed. Saved to:
 20/04/2007 10:27:30 C:\MassLynx Projects\controle2007.PRO\Data\2004MixCalIRMS01.raw
 20/04/2007 10:27:31 Processing sample no 4 ...
 20/04/2007 10:27:33 Kernel stopping inlet script
 20/04/2007 10:27:41 ...processing complete
 20/04/2007 10:54:29 Starting inlet method - Kernel script
 20/04/2007 10:54:31 Opening Sample log file
 20/04/2007 10:54:31 Commencing analysis of 2004MixCalIRMS02
 20/04/2007 10:54:31 Closing ref gas valves and waiting 30 sec
 20/04/2007 11:09:37 Sample log file closed. Saved to:
 20/04/2007 11:09:37 C:\MassLynx Projects\controle2007.PRO\Data\2004MixCalIRMS02.raw
 20/04/2007 11:09:38 Processing sample no 5 ...
 20/04/2007 11:09:40 Kernel stopping inlet script
 20/04/2007 11:09:49 ...processing complete
 20/04/2007 11:10:03 Starting inlet method - Kernel script
 20/04/2007 11:10:06 Opening Sample log file
 20/04/2007 11:10:06 Commencing analysis of 2004MixCalIRMS03
 20/04/2007 11:10:06 Closing ref gas valves and waiting 30 sec
 20/04/2007 11:25:11 C:\MassLynx Projects\controle2007.PRO\Data\2004MixCalIRMS03.raw
 20/04/2007 11:25:12 Processing sample no 6 ...
 20/04/2007 11:25:14 Kernel stopping inlet script
 20/04/2007 11:25:21 ...processing complete
 20/04/2007 11:26:16 Starting inlet method - Kernel script
 20/04/2007 11:26:19 Opening Sample log file
 20/04/2007 11:26:19 Commencing analysis of 2004MixCalAcetate01
 20/04/2007 11:26:19 Closing ref gas valves and waiting 30 sec
 20/04/2007 12:09:15 Sample log file closed. Saved to:
 20/04/2007 12:09:15 C:\MassLynx Projects\controle2007.PRO\Data\2004MixCalAcetate01.raw
 20/04/2007 12:09:16 Processing sample no 7 ...
 20/04/2007 12:09:20 Kernel stopping inlet script
 20/04/2007 12:09:27 ...processing complete
 20/04/2007 12:22:42 Starting inlet method - Kernel script
 20/04/2007 12:22:45 Opening Sample log file
 20/04/2007 12:22:45 Commencing analysis of 2004MixCalAcetate01
 20/04/2007 12:22:45 Closing ref gas valves and waiting 30 sec
 20/04/2007 13:06:48 Sample log file closed. Saved to:

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES CARBONAT

20/04/2007 13:06:48 C:\MassLynx Projects\controle2007.PRO\Data\2004MixCalAcetate01.raw
20/04/2007 13:06:49 Kernel stopping inlet script
20/04/2007 13:06:50 Processing sample no 7 ...
20/04/2007 13:07:03 ...processing complete
20/04/2007 13:07:19 Starting inlet method - Kernel script
20/04/2007 13:07:22 Opening Sample log file
20/04/2007 13:07:22 Commencing analysis of 2004BluIF3
20/04/2007 13:07:22 Closing ref gas valves and waiting 30 sec
20/04/2007 13:51:33 Sample log file closed. Saved to:
20/04/2007 13:51:33 C:\MassLynx Projects\controle2007.PRO\Data\2004BluIF3.raw
20/04/2007 13:51:34 Processing sample no 8 ...
20/04/2007 13:51:38 Kernel stopping inlet script
20/04/2007 13:51:51 ...processing complete
20/04/2007 13:52:07 Starting inlet method - Kernel script
20/04/2007 13:52:09 Opening Sample log file
20/04/2007 13:52:10 Commencing analysis of 2004856F3
20/04/2007 13:52:10 Closing ref gas valves and waiting 30 sec
20/04/2007 14:36:15 Sample log file closed. Saved to:
20/04/2007 14:36:15 C:\MassLynx Projects\controle2007.PRO\Data\2004856F3.raw
20/04/2007 14:36:15 Processing sample no 9 ...
20/04/2007 14:36:19 Kernel stopping inlet script
20/04/2007 14:36:31 ...processing complete
20/04/2007 14:36:52 Starting inlet method - Kernel script
20/04/2007 14:36:55 Opening Sample log file
20/04/2007 14:36:55 Commencing analysis of 2004856F3-2
20/04/2007 14:36:55 Closing ref gas valves and waiting 30 sec
20/04/2007 15:21:06 Sample log file closed. Saved to:
20/04/2007 15:21:06 C:\MassLynx Projects\controle2007.PRO\Data\2004856F3-2.raw
20/04/2007 15:21:07 Processing sample no 10 ...
20/04/2007 15:21:11 Kernel stopping inlet script
20/04/2007 15:21:23 ...processing complete
20/04/2007 15:21:39 Starting inlet method - Kernel script
20/04/2007 15:21:40 Opening Sample log file
20/04/2007 15:21:40 Commencing analysis of 2004BluIF2
20/04/2007 15:21:40 Closing ref gas valves and waiting 30 sec
20/04/2007 16:05:47 Sample log file closed. Saved to:
20/04/2007 16:05:47 C:\MassLynx Projects\controle2007.PRO\Data\2004BluIF2.raw
20/04/2007 16:05:48 Processing sample no 11 ...
20/04/2007 16:05:52 Kernel stopping inlet script
20/04/2007 16:06:03 ...processing complete
20/04/2007 16:06:18 Starting inlet method - Kernel script
20/04/2007 16:06:19 Commencing analysis of 2004856F2
20/04/2007 16:06:19 Closing ref gas valves and waiting 30 sec
20/04/2007 16:50:30 Sample log file closed. Saved to:
20/04/2007 16:50:30 C:\MassLynx Projects\controle2007.PRO\Data\2004856F2.raw
20/04/2007 16:50:31 Processing sample no 12 ...
20/04/2007 16:50:31 Kernel stopping inlet script
20/04/2007 16:50:44 ...processing complete
20/04/2007 16:50:58 Starting inlet method - Kernel script
20/04/2007 16:50:58 Opening Sample log file
20/04/2007 16:50:59 Commencing analysis of 2004BluIF1
20/04/2007 16:50:59 Closing ref gas valves and waiting 30 sec
20/04/2007 17:35:05 Processing sample no 13 ...
20/04/2007 17:35:05 Sample log file closed. Saved to:
20/04/2007 17:35:05 C:\MassLynx Projects\controle2007.PRO\Data\2004BluIF1.raw
20/04/2007 17:35:09 Kernel stopping inlet script
20/04/2007 17:35:19 ...processing complete
20/04/2007 17:35:33 Starting inlet method - Kernel script
20/04/2007 17:35:36 Opening Sample log file

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

20/04/2007 17:35:37 Commencing analysis of 2004856F1
 20/04/2007 17:35:37 Closing ref gas valves and waiting 30 sec
 20/04/2007 18:19:47 Sample log file closed. Saved to:
 20/04/2007 18:19:47 C:\MassLynx Projects\controle2007.PRO\Data\2004856F1.raw
 20/04/2007 18:19:48 Processing sample no 14 ...
 20/04/2007 18:19:53 Kernel stopping inlet script
 20/04/2007 18:20:08 ...processing complete
 20/04/2007 18:20:30 Starting inlet method - Kernel script
 20/04/2007 18:20:33 Opening Sample log file
 20/04/2007 18:20:33 Commencing analysis of 2004Blu2F3
 20/04/2007 18:20:33 Closing ref gas valves and waiting 30 sec
 20/04/2007 19:04:37 Sample log file closed. Saved to:
 20/04/2007 19:04:37 C:\MassLynx Projects\controle2007.PRO\Data\2004Blu2F3.raw
 20/04/2007 19:04:38 Processing sample no 15 ...
 20/04/2007 19:04:38 Kernel stopping inlet script
 20/04/2007 19:04:52 ...processing complete
 20/04/2007 19:05:48 Starting inlet method - Kernel script
 20/04/2007 19:05:52 Opening Sample log file
 20/04/2007 19:05:52 Commencing analysis of 2004425F3
 20/04/2007 19:05:52 Closing ref gas valves and waiting 30 sec
 20/04/2007 19:50:03 Sample log file closed. Saved to:
 20/04/2007 19:50:03 C:\MassLynx Projects\controle2007.PRO\Data\2004425F3.raw
 20/04/2007 19:50:04 Kernel stopping inlet script
 20/04/2007 19:50:04 Processing sample no 16 ...
 20/04/2007 19:50:15 ...processing complete
 20/04/2007 19:50:32 Starting inlet method - Kernel script
 20/04/2007 19:50:33 Opening Sample log file
 20/04/2007 19:50:33 Commencing analysis of 2004Blu2F2
 20/04/2007 19:50:33 Closing ref gas valves and waiting 30 sec
 20/04/2007 20:34:40 Processing sample no 17 ...
 20/04/2007 20:34:40 Sample log file closed. Saved to:
 20/04/2007 20:34:40 C:\MassLynx Projects\controle2007.PRO\Data\2004Blu2F2.raw
 20/04/2007 20:34:44 Kernel stopping inlet script
 20/04/2007 20:34:51 ...processing complete
 20/04/2007 20:35:04 Starting inlet method - Kernel script
 20/04/2007 20:35:05 Opening Sample log file
 20/04/2007 20:35:05 Commencing analysis of 2004425F2
 20/04/2007 20:35:05 Closing ref gas valves and waiting 30 sec
 20/04/2007 21:19:19 Sample log file closed. Saved to:
 20/04/2007 21:19:19 C:\MassLynx Projects\controle2007.PRO\Data\2004425F2.raw
 20/04/2007 21:19:19 Processing sample no 18 ...
 20/04/2007 21:19:22 Kernel stopping inlet script
 20/04/2007 21:19:34 ...processing complete
 20/04/2007 21:19:49 Starting inlet method - Kernel script
 20/04/2007 21:19:50 Opening Sample log file
 20/04/2007 21:19:50 Commencing analysis of 2004Blu2F1
 20/04/2007 21:19:50 Closing ref gas valves and waiting 30 sec
 20/04/2007 22:03:55 Sample log file closed. Saved to:
 20/04/2007 22:03:55 C:\MassLynx Projects\controle2007.PRO\Data\2004Blu2F1.raw
 20/04/2007 22:03:56 Processing sample no 19 ...
 20/04/2007 22:03:57 Kernel stopping inlet script
 20/04/2007 22:04:09 ...processing complete
 20/04/2007 22:04:24 Starting inlet method - Kernel script
 20/04/2007 22:04:25 Opening Sample log file
 20/04/2007 22:04:25 Commencing analysis of 2004425F1
 20/04/2007 22:04:25 Closing ref gas valves and waiting 30 sec
 20/04/2007 22:48:37 Sample log file closed. Saved to:
 20/04/2007 22:48:37 C:\MassLynx Projects\controle2007.PRO\Data\2004425F1.raw
 20/04/2007 22:48:38 Processing sample no 20 ...
 20/04/2007 22:48:42 Kernel stopping inlet script

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

20/04/2007 22:48:52 ...processing complete
 20/04/2007 22:49:06 Starting inlet method - Kernel script
 20/04/2007 22:49:09 Opening Sample log file
 20/04/2007 22:49:10 Commencing analysis of 2004MixCalAcetate02
 20/04/2007 22:49:10 Closing ref gas valves and waiting 30 sec
 20/04/2007 23:33:13 Sample log file closed. Saved to:
 20/04/2007 23:33:13 C:\MassLynx Projects\controle2007.PRO\Data\2004MixCalAcetate02.raw
 20/04/2007 23:33:14 Kernel stopping inlet script
 20/04/2007 23:33:14 Processing sample no 21 ...
 20/04/2007 23:33:26 ...processing complete
 21/04/2007 08:45:20 Starting inlet method - Kernel script
 21/04/2007 08:45:21 Opening Sample log file
 21/04/2007 08:45:31 Starting acquisition
 21/04/2007 08:45:36 Sample log file closed. Saved to:
 21/04/2007 08:45:36 C:\MassLynx Projects\controle2007.PRO\Data\2104stabilite1.raw
 21/04/2007 08:45:38 Kernel stopping inlet script
 21/04/2007 08:47:23 Starting inlet method - Kernel script
 21/04/2007 08:47:24 Opening Sample log file
 21/04/2007 08:47:35 Starting acquisition
 21/04/2007 08:48:14 Sample log file closed. Saved to:
 21/04/2007 08:48:14 C:\MassLynx Projects\controle2007.PRO\Data\2104stabilite1.raw
 21/04/2007 08:48:15 Kernel stopping inlet script
 21/04/2007 08:48:44 Starting inlet method - Kernel script
 21/04/2007 08:48:45 Opening Sample log file
 21/04/2007 08:48:55 Starting acquisition
 21/04/2007 08:59:07 Sample log file closed. Saved to:
 21/04/2007 08:59:07 C:\MassLynx Projects\controle2007.PRO\Data\2104stabilite1.raw
 21/04/2007 08:59:07 Processing sample no 1 ...
 21/04/2007 08:59:07 Kernel stopping inlet script
 21/04/2007 08:59:16 ...processing complete
 21/04/2007 08:59:19 Starting inlet method - Kernel script
 21/04/2007 08:59:20 Opening Sample log file
 21/04/2007 08:59:30 Starting acquisition
 21/04/2007 09:09:42 Processing sample no 2 ...
 21/04/2007 09:09:42 Sample log file closed. Saved to:
 21/04/2007 09:09:42 C:\MassLynx Projects\controle2007.PRO\Data\2104stabilite2.raw
 21/04/2007 09:09:43 Kernel stopping inlet script
 21/04/2007 09:09:52 ...processing complete
 21/04/2007 09:09:55 Starting inlet method - Kernel script
 21/04/2007 09:09:58 Opening Sample log file
 21/04/2007 09:10:09 Starting acquisition
 21/04/2007 09:20:20 Sample log file closed. Saved to:
 21/04/2007 09:20:20 C:\MassLynx Projects\controle2007.PRO\Data\2104stabilite3.raw
 21/04/2007 09:20:21 Processing sample no 3 ...
 21/04/2007 09:20:25 Kernel stopping inlet script
 21/04/2007 09:20:33 ...processing complete
 21/04/2007 09:25:31 Starting inlet method - Kernel script
 21/04/2007 09:25:32 Opening Sample log file
 21/04/2007 09:25:32 Commencing analysis of 2104MixCalIRMS01
 21/04/2007 09:25:32 Closing ref gas valves and waiting 30 sec
 21/04/2007 09:40:44 Sample log file closed. Saved to:
 21/04/2007 09:40:44 C:\MassLynx Projects\controle2007.PRO\Data\2104MixCalIRMS01.raw
 21/04/2007 09:40:45 Processing sample no 4 ...
 21/04/2007 09:40:48 Kernel stopping inlet script
 21/04/2007 09:40:56 ...processing complete
 21/04/2007 09:41:10 Starting inlet method - Kernel script
 21/04/2007 09:41:11 Opening Sample log file
 21/04/2007 09:41:11 Commencing analysis of 2104MixCalIRMS02
 21/04/2007 09:41:11 Closing ref gas valves and waiting 30 sec
 21/04/2007 09:56:19 Sample log file closed. Saved to:

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

21/04/2007 09:56:19 C:\MassLynx Projects\controle2007.PRO\Data\2104MixCalIRMS02.raw
21/04/2007 09:56:20 Processing sample no 5 ...
21/04/2007 09:56:21 Kernel stopping inlet script
21/04/2007 09:56:33 ...processing complete
21/04/2007 09:56:47 Starting inlet method - Kernel script
21/04/2007 09:56:51 Opening Sample log file
21/04/2007 09:56:51 Commencing analysis of 2104MixCalIRMS03
21/04/2007 09:56:51 Closing ref gas valves and waiting 30 sec
21/04/2007 10:11:56 Sample log file closed. Saved to:
21/04/2007 10:11:56 C:\MassLynx Projects\controle2007.PRO\Data\2104MixCalIRMS03.raw
21/04/2007 10:11:56 Processing sample no 6 ...
21/04/2007 10:11:59 Kernel stopping inlet script
21/04/2007 10:12:08 ...processing complete
21/04/2007 10:16:19 Starting inlet method - Kernel script
21/04/2007 10:16:20 Opening Sample log file
21/04/2007 10:16:20 Commencing analysis of 2104MixCalIRMS01
21/04/2007 10:16:20 Closing ref gas valves and waiting 30 sec
21/04/2007 10:31:27 Sample log file closed. Saved to:
21/04/2007 10:31:27 C:\MassLynx Projects\controle2007.PRO\Data\2104MixCalIRMS01.raw
21/04/2007 10:31:27 Processing sample no 4 ...
21/04/2007 10:31:30 Kernel stopping inlet script
21/04/2007 10:31:37 ...processing complete
21/04/2007 10:31:51 Starting inlet method - Kernel script
21/04/2007 10:31:52 Opening Sample log file
21/04/2007 10:31:52 Commencing analysis of 2104MixCalIRMS02
21/04/2007 10:31:52 Closing ref gas valves and waiting 30 sec
21/04/2007 10:47:05 Sample log file closed. Saved to:
21/04/2007 10:47:05 C:\MassLynx Projects\controle2007.PRO\Data\2104MixCalIRMS02.raw
21/04/2007 10:47:06 Processing sample no 5 ...
21/04/2007 10:47:08 Kernel stopping inlet script
21/04/2007 10:47:18 ...processing complete
21/04/2007 10:47:34 Starting inlet method - Kernel script
21/04/2007 10:47:37 Opening Sample log file
21/04/2007 10:47:37 Commencing analysis of 2104MixCalIRMS03
21/04/2007 10:47:37 Closing ref gas valves and waiting 30 sec
21/04/2007 11:00:53 Sample log file closed. Saved to:
21/04/2007 11:00:53 C:\MassLynx Projects\controle2007.PRO\Data\2104MixCalIRMS03.raw
21/04/2007 11:00:53 Processing sample no 6 ...
21/04/2007 11:00:56 Kernel stopping inlet script
21/04/2007 11:01:02 ...processing complete
21/04/2007 11:08:01 Starting inlet method - Kernel script
21/04/2007 11:08:04 Opening Sample log file
21/04/2007 11:08:04 Commencing analysis of 2104MixCalAcetate01
21/04/2007 11:08:04 Closing ref gas valves and waiting 30 sec
21/04/2007 11:52:08 Sample log file closed. Saved to:
21/04/2007 11:52:08 C:\MassLynx Projects\controle2007.PRO\Data\2104MixCalAcetate01.raw
21/04/2007 11:52:09 Processing sample no 7 ...
21/04/2007 11:52:13 Kernel stopping inlet script
21/04/2007 11:52:23 ...processing complete
21/04/2007 12:12:29 Starting inlet method - Kernel script
21/04/2007 12:12:32 Opening Sample log file
21/04/2007 12:12:32 Commencing analysis of 2104Blu1F3
21/04/2007 12:12:32 Closing ref gas valves and waiting 30 sec
21/04/2007 12:56:43 Sample log file closed. Saved to:
21/04/2007 12:56:43 C:\MassLynx Projects\controle2007.PRO\Data\2104Blu1F3.raw
21/04/2007 12:56:44 Processing sample no 8 ...
21/04/2007 12:56:48 Kernel stopping inlet script
21/04/2007 12:56:59 ...processing complete
21/04/2007 12:57:14 Starting inlet method - Kernel script
21/04/2007 12:57:17 Opening Sample log file

COPIE
CONFORME
ET FORMULAIRE

21/04/2007 12:57:17 Commencing analysis of 2104427F3
 21/04/2007 12:57:17 Closing ref gas valves and waiting 30 sec
 21/04/2007 13:41:22 Sample log file closed. Saved to:
 21/04/2007 13:41:22 C:\MassLynx Projects\controle2007.PRO\Data\2104427F3.raw
 21/04/2007 13:41:22 Processing sample no 9 ...
 21/04/2007 13:41:27 Kernel stopping inlet script
 21/04/2007 13:41:34 ...processing complete
 21/04/2007 13:41:50 Starting inlet method - Kernel script
 21/04/2007 13:41:51 Opening Sample log file
 21/04/2007 13:41:51 Commencing analysis of 2104Blu1F2
 21/04/2007 13:41:51 Closing ref gas valves and waiting 30 sec
 21/04/2007 14:26:05 Sample log file closed. Saved to:
 21/04/2007 14:26:05 C:\MassLynx Projects\controle2007.PRO\Data\2104Blu1F2.raw
 21/04/2007 14:26:05 Kernel stopping inlet script
 21/04/2007 14:26:05 Processing sample no 10 ...
 21/04/2007 14:26:19 ...processing complete
 21/04/2007 14:26:33 Starting inlet method - Kernel script
 21/04/2007 14:26:36 Opening Sample log file
 21/04/2007 14:26:36 Commencing analysis of 2104427F2
 21/04/2007 14:26:36 Closing ref gas valves and waiting 30 sec
 21/04/2007 15:10:41 Sample log file closed. Saved to:
 21/04/2007 15:10:41 C:\MassLynx Projects\controle2007.PRO\Data\2104427F2.raw
 21/04/2007 15:10:41 Processing sample no 11 ...
 21/04/2007 15:10:46 Kernel stopping inlet script
 21/04/2007 15:10:55 ...processing complete
 21/04/2007 15:49:15 Starting inlet method - Kernel script
 21/04/2007 15:49:16 Opening Sample log file
 21/04/2007 15:49:16 Commencing analysis of 2104Blu1F1
 21/04/2007 15:49:16 Closing ref gas valves and waiting 30 sec
 21/04/2007 16:33:29 Sample log file closed. Saved to:
 21/04/2007 16:33:29 C:\MassLynx Projects\controle2007.PRO\Data\2104Blu1F1.raw
 21/04/2007 16:33:29 Processing sample no 12 ...
 21/04/2007 16:33:34 Kernel stopping inlet script
 21/04/2007 16:33:44 ...processing complete
 21/04/2007 16:33:58 Starting inlet method - Kernel script
 21/04/2007 16:34:01 Opening Sample log file
 21/04/2007 16:34:01 Commencing analysis of 2104427F1
 21/04/2007 16:34:01 Closing ref gas valves and waiting 30 sec
 21/04/2007 17:18:05 Sample log file closed. Saved to:
 21/04/2007 17:18:05 C:\MassLynx Projects\controle2007.PRO\Data\2104427F1.raw
 21/04/2007 17:18:05 Processing sample no 13 ...
 21/04/2007 17:18:05 Kernel stopping inlet script
 21/04/2007 17:18:18 ...processing complete
 21/04/2007 18:06:46 Starting inlet method - Kernel script
 21/04/2007 18:06:49 Opening Sample log file
 21/04/2007 18:06:49 Commencing analysis of 2104Blu2F3
 21/04/2007 18:06:49 Closing ref gas valves and waiting 30 sec
 21/04/2007 18:51:00 Sample log file closed. Saved to:
 21/04/2007 18:51:00 C:\MassLynx Projects\controle2007.PRO\Data\2104Blu2F3.raw
 21/04/2007 18:51:01 Kernel stopping inlet script
 21/04/2007 18:51:01 Processing sample no 14 ...
 21/04/2007 18:51:19 ...processing complete
 21/04/2007 18:51:34 Starting inlet method - Kernel script
 21/04/2007 18:51:37 Opening Sample log file
 21/04/2007 18:51:37 Commencing analysis of 2104865F3
 21/04/2007 18:51:37 Closing ref gas valves and waiting 30 sec
 21/04/2007 19:35:42 Sample log file closed. Saved to:
 21/04/2007 19:35:42 C:\MassLynx Projects\controle2007.PRO\Data\2104865F3.raw
 21/04/2007 19:35:43 Processing sample no 15 ...
 21/04/2007 19:35:47 Kernel stopping inlet script

COPIE CERTIFIÉE
 CONFORME DISPOSITIF
 ET FORMULAIRES CARBONÉ

21/04/2007 19:35:55 ...processing complete
 21/04/2007 19:36:15 Starting inlet method - Kernel script
 21/04/2007 19:36:16 Opening Sample log file
 21/04/2007 19:36:16 Commencing analysis of 2104Blu2F2
 21/04/2007 19:36:16 Closing ref gas valves and waiting 30 sec
 21/04/2007 20:20:30 Sample log file closed. Saved to:
 21/04/2007 20:20:30 C:\MassLynx Projects\controle2007.PRO\Data\2104Blu2F2.raw
 21/04/2007 20:20:30 Processing sample no 16 ...
 21/04/2007 20:20:35 Kernel stopping inlet script
 21/04/2007 20:20:43 ...processing complete
 21/04/2007 20:20:57 Starting inlet method - Kernel script
 21/04/2007 20:20:58 Opening Sample log file
 21/04/2007 20:20:58 Commencing analysis of 2104865F2
 21/04/2007 20:20:58 Closing ref gas valves and waiting 30 sec
 21/04/2007 21:05:03 Sample log file closed. Saved to:
 21/04/2007 21:05:03 C:\MassLynx Projects\controle2007.PRO\Data\2104865F2.raw
 21/04/2007 21:05:04 Processing sample no 17 ...
 21/04/2007 21:05:09 Kernel stopping inlet script
 21/04/2007 21:05:21 ...processing complete
 21/04/2007 21:05:35 Starting inlet method - Kernel script
 21/04/2007 21:05:36 Opening Sample log file
 21/04/2007 21:05:36 Commencing analysis of 2104Blu2F1
 21/04/2007 21:05:36 Closing ref gas valves and waiting 30 sec
 21/04/2007 21:49:49 Sample log file closed. Saved to:
 21/04/2007 21:49:49 C:\MassLynx Projects\controle2007.PRO\Data\2104Blu2F1.raw
 21/04/2007 21:49:49 Processing sample no 18 ...
 21/04/2007 21:49:54 Kernel stopping inlet script
 21/04/2007 21:50:01 ...processing complete
 21/04/2007 21:50:16 Starting inlet method - Kernel script
 21/04/2007 21:50:17 Opening Sample log file
 21/04/2007 21:50:17 Commencing analysis of 2104865F1
 21/04/2007 21:50:17 Closing ref gas valves and waiting 30 sec
 21/04/2007 22:34:22 Sample log file closed. Saved to:
 21/04/2007 22:34:22 C:\MassLynx Projects\controle2007.PRO\Data\2104865F1.raw
 21/04/2007 22:34:23 Processing sample no 19 ...
 21/04/2007 22:34:23 Kernel stopping inlet script
 21/04/2007 22:34:36 ...processing complete
 21/04/2007 22:34:49 Starting inlet method - Kernel script
 21/04/2007 22:34:52 Opening Sample log file
 21/04/2007 22:34:52 Commencing analysis of 2104MixCalAcetate02
 21/04/2007 22:34:52 Closing ref gas valves and waiting 30 sec
 21/04/2007 23:19:02 Sample log file closed. Saved to:
 21/04/2007 23:19:02 C:\MassLynx Projects\controle2007.PRO\Data\2104MixCalAcetate02.raw
 21/04/2007 23:19:02 Kernel stopping inlet script
 21/04/2007 23:19:03 Processing sample no 20 ...
 21/04/2007 23:19:15 ...processing complete
 22/04/2007 11:47:51 Starting inlet method - Kernel script
 22/04/2007 11:47:52 Opening Sample log file
 22/04/2007 11:48:03 Starting acquisition
 22/04/2007 11:58:14 Processing sample no 1 ...
 22/04/2007 11:58:15 Sample log file closed. Saved to:
 22/04/2007 11:58:15 C:\MassLynx Projects\controle2007.PRO\Data\2204stabilite1.raw
 22/04/2007 11:58:17 Kernel stopping inlet script
 22/04/2007 11:58:28 ...processing complete
 22/04/2007 11:58:31 Starting inlet method - Kernel script
 22/04/2007 11:58:32 Opening Sample log file
 22/04/2007 11:58:42 Starting acquisition
 22/04/2007 12:08:54 Sample log file closed. Saved to:
 22/04/2007 12:08:54 C:\MassLynx Projects\controle2007.PRO\Data\2204stabilite2.raw
 22/04/2007 12:08:54 Processing sample no 2 ...

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

22/04/2007 12:08:59 Kernel stopping inlet script
 22/04/2007 12:09:07 ...processing complete
 22/04/2007 12:09:10 Starting inlet method - Kernel script
 22/04/2007 12:09:11 Opening Sample log file
 22/04/2007 12:09:21 Starting acquisition
 22/04/2007 12:19:33 Sample log file closed. Saved to:
 22/04/2007 12:19:33 C:\MassLynx Projects\controle2007.PRO\Data\2204stabilite3.raw
 22/04/2007 12:19:33 Processing sample no 3 ...
 22/04/2007 12:19:36 Kernel stopping inlet script
 22/04/2007 12:19:46 ...processing complete
 22/04/2007 12:21:19 Starting inlet method - Kernel script
 22/04/2007 12:21:22 Opening Sample log file
 22/04/2007 12:21:22 Commencing analysis of 2204MixCalIRMS01
 22/04/2007 12:21:22 Closing ref gas valves and waiting 30 sec
 22/04/2007 12:36:33 Sample log file closed. Saved to:
 22/04/2007 12:36:33 C:\MassLynx Projects\controle2007.PRO\Data\2204MixCalIRMS01.raw
 22/04/2007 12:36:34 Processing sample no 4 ...
 22/04/2007 12:36:36 Kernel stopping inlet script
 22/04/2007 12:36:48 ...processing complete
 22/04/2007 12:37:09 Starting inlet method - Kernel script
 22/04/2007 12:37:10 Opening Sample log file
 22/04/2007 12:37:10 Commencing analysis of 2204MixCalIRMS02
 22/04/2007 12:37:10 Closing ref gas valves and waiting 30 sec
 22/04/2007 12:52:17 Sample log file closed. Saved to:
 22/04/2007 12:52:17 C:\MassLynx Projects\controle2007.PRO\Data\2204MixCalIRMS02.raw
 22/04/2007 12:52:17 Processing sample no 5 ...
 22/04/2007 12:52:20 Kernel stopping inlet script
 22/04/2007 12:52:30 ...processing complete
 22/04/2007 12:52:44 Starting inlet method - Kernel script
 22/04/2007 12:52:45 Opening Sample log file
 22/04/2007 12:52:45 Commencing analysis of 2204MixCalIRMS03
 22/04/2007 12:52:45 Closing ref gas valves and waiting 30 sec
 22/04/2007 13:07:52 Sample log file closed. Saved to:
 22/04/2007 13:07:52 C:\MassLynx Projects\controle2007.PRO\Data\2204MixCalIRMS03.raw
 22/04/2007 13:07:52 Processing sample no 6 ...
 22/04/2007 13:07:55 Kernel stopping inlet script
 22/04/2007 13:08:06 ...processing complete
 22/04/2007 13:08:58 Starting inlet method - Kernel script
 22/04/2007 13:09:01 Opening Sample log file
 22/04/2007 13:09:02 Commencing analysis of 2204MixCalAcetate01
 22/04/2007 13:09:02 Closing ref gas valves and waiting 30 sec
 22/04/2007 13:53:00 Sample log file closed. Saved to:
 22/04/2007 13:53:00 C:\MassLynx Projects\controle2007.PRO\Data\2204MixCalAcetate01.raw
 22/04/2007 13:53:00 Processing sample no 7 ...
 22/04/2007 13:53:01 Kernel stopping inlet script
 22/04/2007 13:53:13 ...processing complete
 22/04/2007 13:55:10 Starting inlet method - Kernel script
 22/04/2007 13:55:13 Opening Sample log file
 22/04/2007 13:55:13 Commencing analysis of 2204MixCalAcetate01
 22/04/2007 13:55:13 Closing ref gas valves and waiting 30 sec
 22/04/2007 13:55:43 IsoPrime is not waiting to acquire - WaitTillRunning
 22/04/2007 13:55:43 Sample log file closed. Saved to:
 22/04/2007 13:55:43 C:\MassLynx Projects\controle2007.PRO\Data\2204MixCalAcetate01.raw
 22/04/2007 13:55:44 Kernel stopping inlet script
 22/04/2007 13:57:25 Starting inlet method - Kernel script
 22/04/2007 13:57:28 Opening Sample log file
 22/04/2007 13:57:28 Commencing analysis of 2204MixCalAcetate01
 22/04/2007 13:57:28 Closing ref gas valves and waiting 30 sec
 22/04/2007 14:41:39 Sample log file closed. Saved to:
 22/04/2007 14:41:39 C:\MassLynx Projects\controle2007.PRO\Data\2204MixCalAcetate01.raw

COPIE CERTIFIÉE
 CONFORME DES DONNÉES
 ET FORMULAIRES ORIGINAUX

204

22/04/2007 14:41:39 Processing sample no 7 ...
 22/04/2007 14:41:44 Kernel stopping inlet script
 22/04/2007 14:41:54 ...processing complete
 22/04/2007 14:42:45 Starting inlet method - Kernel script
 22/04/2007 14:42:48 Opening Sample log file
 22/04/2007 14:42:48 Commencing analysis of 2204Blu1F3
 22/04/2007 14:42:48 Closing ref gas valves and waiting 30 sec
 22/04/2007 15:25:16 Sample log file closed. Saved to:
 22/04/2007 15:25:16 C:\MassLynx Projects\controle2007.PRO\Data\2204Blu1F3.raw
 22/04/2007 15:25:16 Processing sample no 8 ...
 22/04/2007 15:25:17 Kernel stopping inlet script
 22/04/2007 15:25:31 ...processing complete
 22/04/2007 15:25:47 Starting inlet method - Kernel script
 22/04/2007 15:25:50 Opening Sample log file
 22/04/2007 15:25:51 Commencing analysis of 2204424F3
 22/04/2007 15:25:51 Closing ref gas valves and waiting 30 sec
 22/04/2007 16:09:52 Sample log file closed. Saved to:
 22/04/2007 16:09:52 C:\MassLynx Projects\controle2007.PRO\Data\2204424F3.raw
 22/04/2007 16:09:53 Processing sample no 9 ...
 22/04/2007 16:09:54 Kernel stopping inlet script
 22/04/2007 16:10:07 ...processing complete
 22/04/2007 16:14:01 Starting inlet method - Kernel script
 22/04/2007 16:14:02 Opening Sample log file
 22/04/2007 16:14:02 Commencing analysis of 2204Blu1F2
 22/04/2007 16:14:03 Closing ref gas valves and waiting 30 sec
 22/04/2007 16:58:10 Sample log file closed. Saved to:
 22/04/2007 16:58:10 C:\MassLynx Projects\controle2007.PRO\Data\2204Blu1F2.raw
 22/04/2007 16:58:10 Processing sample no 10 ...
 22/04/2007 16:58:15 Kernel stopping inlet script
 22/04/2007 16:58:25 ...processing complete
 22/04/2007 16:58:38 Starting inlet method - Kernel script
 22/04/2007 16:58:39 Opening Sample log file
 22/04/2007 16:58:39 Commencing analysis of 2204424F2
 22/04/2007 16:58:39 Closing ref gas valves and waiting 30 sec
 22/04/2007 17:42:53 Sample log file closed. Saved to:
 22/04/2007 17:42:53 C:\MassLynx Projects\controle2007.PRO\Data\2204424F2.raw
 22/04/2007 17:42:53 Processing sample no 11 ...
 22/04/2007 17:42:58 Kernel stopping inlet script
 22/04/2007 17:43:07 ...processing complete
 22/04/2007 17:43:20 Starting inlet method - Kernel script
 22/04/2007 17:43:21 Opening Sample log file
 22/04/2007 17:43:21 Commencing analysis of 2204Blu1F1
 22/04/2007 17:43:21 Closing ref gas valves and waiting 30 sec
 22/04/2007 18:27:27 Sample log file closed. Saved to:
 22/04/2007 18:27:27 C:\MassLynx Projects\controle2007.PRO\Data\2204Blu1F1.raw
 22/04/2007 18:27:28 Processing sample no 12 ...
 22/04/2007 18:27:32 Kernel stopping inlet script
 22/04/2007 18:27:42 ...processing complete
 22/04/2007 18:28:02 Starting inlet method - Kernel script
 22/04/2007 18:28:03 Opening Sample log file
 22/04/2007 18:28:03 Commencing analysis of 2204424F1
 22/04/2007 18:28:03 Closing ref gas valves and waiting 30 sec
 22/04/2007 19:12:16 Sample log file closed. Saved to:
 22/04/2007 19:12:16 C:\MassLynx Projects\controle2007.PRO\Data\2204424F1.raw
 22/04/2007 19:12:16 Processing sample no 13 ...
 22/04/2007 19:12:21 Kernel stopping inlet script
 22/04/2007 19:12:31 ...processing complete
 22/04/2007 19:12:45 Starting inlet method - Kernel script
 22/04/2007 19:12:48 Opening Sample log file
 22/04/2007 19:12:48 Commencing analysis of 2204MixCalAcetate02

CONFIDENTIAL
 PROPRIETARY
 INFORMATION

22/04/2007 19:12:48 Closing ref gas valves and waiting 30 sec
22/04/2007 19:56:52 Sample log file closed. Saved to:
22/04/2007 19:56:52 C:\MassLynx Projects\controle2007.PRO\Data\2204MixCalAcetate02.raw
22/04/2007 19:56:52 Kernel stopping inlet script
22/04/2007 19:56:52 Processing sample no 14 ...
22/04/2007 19:57:08 ...processing complete

COPIE CERTIFIÉE
CONFORME DES DONNÉES
ET FORMULAIRES ORIGINAUX

GDC01075

REPROCESSING OF ELECTRONIC DATAFILES

Chatenay-Malabry (Paris, France) May 4-5 2006

Summary of Operation

On May 4th 2007, at 1.00 pm, technical experts of Mr Landis and of the USADA have met in the presence of an independent expert of the Panel at the Laboratoire National du Depistage du Dopage (LNDD) in Chatenay Malabry, to carry out the reprocessing of the Electronic Data Files (EDFs) copied on April 26th 2007.

The following people were present:

Dr. Simon Davis (scientific expert of Mr. Landis),¹ Dr. Thomas Brenna, Dr. Jeanine Jumeau (scientific experts of the USADA), Dr. Jacques de Ceaurriz (Director of the LNDD), Dr. Corinne Buisson (Responsible for the IRMS Department of the LNDD) Dr. Francesco Botrè (Independent expert for the Panel).

Before proceeding with the re-processing of the EDFs, Drs. de Ceaurriz and Buisson were asked to supply additional information on the process of backup of electronic data, either in general and with specific reference to those data copied on the three CD-ROMs on April 26th 2006 and still under the custody of the independent expert of the Panel. This mainly to further clarify the meaning of the following statement, reported at page 1 of the document "Copy of Electronic Datafiles – Summary of Operation" dated April 26th 2007:

"The datafiles of the IRMS analysis of the "A" and "B" sample of stage 17 were not copied directly from the hard disk of the instrument, but from a CD-ROM on which they had been previously backed up, as part of the internal procedures of the laboratory, by the personnel of the LNDD."

The following information was obtained:

- The process of backup of the datafiles from the internal memory of the old Isoprime (S.N. JA 010), i.e. the instrument on which the instrumental analysis of the "A" and "B" samples of stage 17 was performed, is periodically carried out manually, under the responsibility of the LNDD. In practice, these operations are performed by an external company that has a specific contract with the LNDD.
- Specifically, the original backup of the data concerning the analysis of the "A" and "B" samples of stage 17 was carried out on October 31th 2006. This is the process that was performed by removing the internal hard disk of the instrument, connecting it to a PC with a CD-writer and then re-installing the hard disk back in place inside the instrument. The data were stored on two CD-ROMs, labelled as "Backup du 31/10/2006 Data: 010206 → 251006 CD1" and "Backup du 31/10/2006 Data: 010206 → 251006 CD1". These are to be considered the two "master" CDs.
- The data stored on the two above mentioned master CDs were then copied, for practical reasons (i.e. to allow a faster retrieval of information), on more, additional CDs. Particularly, the data concerning the analysis of the "A" sample of stage 17 had been archived, together with other data files, also into another CD, labelled as "Isoprime I Data Juin 06 à 31/07/06 (créé le 30/01/07)".
- Finally, the data concerning the analysis of the "A" and "B" samples of stage 17 were also copied on another CD, labelled as "Isop 1 23/07/06 04/08/06"; this last CD, produced in the morning of April 26th 2007, contained only the data of the

two samples without any additional data (e.g. those of the assays to assess the linearity).

- The CD labelled as "Electronic Data Files Stage 17 A+B + Linearity", produced in the afternoon of April 26th 2007 at the presence of the technical experts of the Parties and of the independent expert of the Panel, contained copies of files stored on both the CD labelled as "Isop 1 23/07/06 04/08/06" (data files of samples "A" and "B" of stage 17) and on the CD labelled as "Isoprime I Data Juin 06 à 31/07/06 (créé le 30/01/07)" (data files of the linearity assays).

The above back-up history was documented by obtaining hardcopies of the list of folders/files stored on each one of the above mentioned CD-ROMs.

Prior to begin the extraction of the EDFs, the Parties discussed on which were the files to be reprocessed, whether only the files of the sample (A and B) collected on the occasion of the stage 17, or also those referring to the 10 blind "B" samples analyzed by GC-IRMS in the period from April 17th to April 22nd 2007. The discussion also involved the request of Mr. Landis' expert to access the logfile copied on the CD-ROM labeled as "Logfile" on the occasion of the previous meeting at the LNDD, on April 26th 2006. According to the instructions received by the President of the Panel, Mr. Patrice Brunet, in his email message of May 3rd 2007, Dr. Botrè tried without success to contact by phone Mr. Richard Campbell, and left a message on his mobile phone voicemail. In order to optimize the timeframe of operation, it was agreed to go on anyway with the reprocessing of the EDFs of the "A" and "B" samples from stage 17 first, and to go back to the issue of the 10 blind samples datafiles and of the logfile later on.

The sealed envelope containing the CD-ROM labelled as "Electronic Data Files Stage 17 A+B + Linearity" was then opened by Dr. Botrè and, at 3.05 pm, all present moved from the meeting room to the laboratory room hosting the GC-IRMS instruments.

The reprocessing of the EDFs was first performed on the same instrument used for the original analysis of the "A" and "B" samples of stage 17. The process was performed in the presence of Drs. Davis, Brenna, Juneau, Buisson and Botrè, while Dr. de Caurriz was not constantly present in the GC-IRMS room.

To carry out the reprocessing of the files it was necessary to copy back the files from the CD to the internal memory of the instrument. It was indeed not possible to load and reprocess the data directly from the CD-ROM. Two new folders ("230706" and "040806") were then created on the internal memory of the instruments, and the relevant files were copied inside these two folders from the CD-ROM. A printout of the list of folders contained in the data directory of the internal hard disk of the instrument was produced immediately before and right after the copying process took place.

Once the files were copied, it was possible to start with their reprocessing. It was agreed that an analyst of the LNDD (namely, the same person who originally processed the data on the occasion respectively of the "A" and "B" analysis, according to the information reported on the Laboratory Documentation Packages) would have operated the computer to carry out the reprocessing of the datafiles, under the responsibility and at the presence of the independent expert of the Panel and at the presence of the experts of the Parties.

It was agreed that three outputs had to be produced for each file: (a) a first one, applying the automatic subtraction of the background; (b) a second one, manually

subtracting the background; (c) a third one, with no background subtraction. Each output consists of two pages, a data page ("Data Processing Results") and a graphical page. The output of each file is then represented by 6 (six) printed pages.

It was agreed to start with the reprocessing of the datafiles of the "A" sample, i.e. with those copied in the folder "230706".

The reprocessing of the files started at 3.45pm. The reprocessing of the datafiles of the "A" sample was completed without comparing the outputs with those reported in the Laboratory Documentation Packages.

At 7.00pm, it was agreed to suspend the operation to continue on the following day.

It was agreed to delete the files (both those just reprocessed and those still to be reprocessed) copied on the internal hard disk of the instrument. A printout of the new list of folders in the data directory was also produced.

The operations ended at 7.30pm. It was agreed to continue the operation on the following day, May 5th, at 9.00am.

The operations started again at 9.15am on May 5th 2007. In a preliminary meeting, it was agreed to complete the process suspended the day before. All the presents moved again to the laboratory room hosting the GC-IRMS instruments at 9.30am. The files not yet reprocessed were copied again on the internal hard disk of the instrument.

The above files were then reprocessed, starting with the EDFs of sample "B", then proceeding with the two sets of the stability runs (one for the "B" and one for the "A" sample), and with the two sets of linearity runs originally performed on June 26th 2006 and on July 31st 2006. Also in this case the reprocessing of the datafiles was completed without comparing the outputs with those reported in the Laboratory Documentation Packages.

After all the above processes were completed, the data were deleted again from the internal hard disk of the instrument.

At 11.20am it was agreed to start the reprocessing of the EDFs on the new instrument. The conversion of the files was carried out by Dr. Buisson, under the supervision of the independent expert of the Panel and at the presence of the technical experts of the parties. A new directory ("Reprocessing050507") was created on the internal hard disk of the instrument and the procedure outlined in the document "07-04-29 EDF Instructions to Dr. Botrè" was followed. The files were successfully converted and saved into the new directory; nonetheless, it was not possible to reprocess them.

Davis explained that this could be due to the lack of some "support" files that are automatically generated during the analysis of real samples and that, consequently, were not present in the new directory. To proceed with the reprocessing of the datafiles, it was agreed to copy, from other data directories into the newly created directory, also those support files. This procedure allowed to open the files under the software of the instrument and to reprocess them for the first time by this instrument.

It was agreed reprocess all those files that had just been reprocessed by the original instrument. The reprocessing of the files was carried out by the same two analysts who had just reprocessed the data on the original instrument.

The file were reprocessed following the routine procedure of the laboratory for the analysis of real samples. Two printouts were produced to show the routine processing parameters of the laboratory, which were applied also for the reprocessing of the datafiles.

It was possible to complete the reprocessing of all files, apart from the datafile "data010" (respective to the blank urine fraction 3 of the "B" analysis), for which an error message appeared. This is documented by two printouts of the screenshot.

At 1.45pm the reprocessing of the datafiles on the new instrument was completed. All the presents left the GC-IRMS laboratory and went to the meeting room.

At this point, Dr. Davis stated that the reprocessing of the datafiles of the 10 blind samples was no longer necessary. It was therefore agreed not to open the sealed envelope containing the corresponding CD-ROM, which remained under the custody of the independent observer. It was no longer necessary to contact the Panel for this issue.

With respect to the issue of the logfile – copied on the CD-ROM labelled as "Logfile" on April 26th 2007 – Dr. Botrè felt it necessary to ask for the opinion of the Panel, also to decide whether or not to supply copies of the printouts produced during the 2-days operation to the Parties. To this respect, Dr. Botrè repeatedly tried to contact – sequentially – Mr. Campbell, Mr. McLaren and Mr. Brunet, without success. At around 15.35pm Mr. McLaren called back. The following indications were supplied to Dr. Botrè: (1) the Parties could be given copies of the printouts, provided it was possible to clearly mark all the copies as originals; (2) it was also agreed that it was not possible to printout the content of the logfile and to give it to the Parties its entirety, since it contained confidential information regarding the activity of the lab for samples other than Mr. Landis'. It was agreed to cut-and-copy only the portion regarding the six days (April 17-22) in which the analysis of the 10 blind samples took place.

A total of 206 printed pages (numbered from 1 to 206) were produced during the two-days of operation. The original copy is under the custody of Dr. Botrè. The other three copies, with every page stamped in red as "Copie certifiée conforme des données et formulaires originaux", were distributed to the Parties and to the LNDD.

The operation ended at 5.20 pm.

Chatenay-Malabry, May 5th 2007.

Dr. Simon Davis

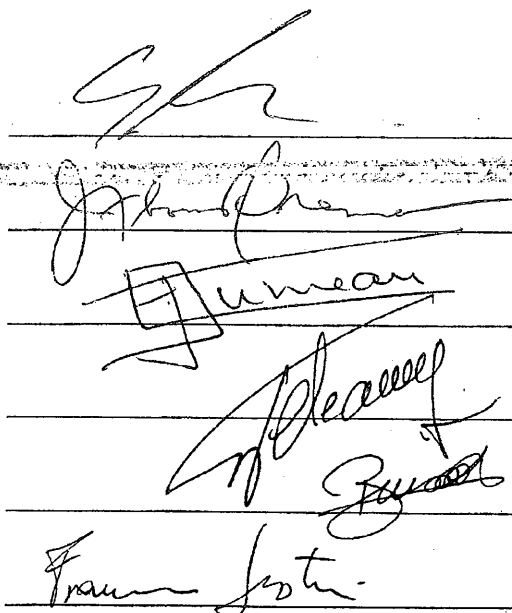
Dr. Thomas Brenna

Dr. Jeanine Jumeau

Dr. Jacques de Ceaurriz

Dr. Corinne Buisson

Dr. Francesco Botrè





Personal Cycling Team

Abingdon AE (Ehntal) CH8894 (Switzerland), Tel: +41 (0)55 264 70 00 Fax: +41 (0)55 264 70 11



Le Tour 2006: Daily Analysis Report

Stage: **Strasbourg to Strasbourg, 184.5 km** Sunday, July 2nd

Floyd Landis

Temp: 84 °F / 29 °C Humidity: 45 % Heat Index: 84.1 °F / 28.97 °C

Stage Results: 184.5 km 44.3 km/hr 27.3 km/hr

GC Results: 179.6 km 35.0 km/hr 44.3 km/hr 27.3 km/hr

Place: 46 Time: 4 hrs 10 min 0 sec Gap: 0 min 0 sec

Place: 9 Time: 4 hrs 18 min 26 sec Gap: 0 hrs 0 min 9 sec

Race Notes: Today the guys just cruised. It was on the warm side and the racing was a bit nervous but all in all it was a pretty chill day for all involved. No comment from Floyd except that it was really easy.

Power, RPE, Heart Rate, Work, RPE vs. Power, HR vs. Power

| | | Power when Moving | | Power when Pedaling | | Strain | | Work or Stress in Kjoules From: | | | RPE | HR |
|-----------|----------|-------------------|----------|---------------------|----------|--------|----|---------------------------------|-------|----|------|-----|
| | | Watts | Watts/kg | Watts | Watts/kg | RPE | HR | Power | RPE | HR | Pwr | Pwr |
| Today | Today | 205 | 2.92 | 250 | 3.56 | 3.5 | NA | 3,075 | 3,113 | NA | 1.01 | NA |
| | Tour Avs | | | | | | | | | | | |
| | Tour Min | | | | | | | | | | | |
| | Tour Max | | | | | | | | | | | |
| Yesterday | Today | 291 | 2.83 | 256 | 3.6 | 4.4 | NA | 3815 | 3644 | NA | 1.21 | |
| | Tour Avs | | | | | | | | | | | |
| | Tour Min | | | | | | | | | | | |
| | Tour Max | | | | | | | | | | | |

Power Distribution:

| | | Zero Watts | | Per Kilogram of Body Weight | | | | | | | | | | | | | | Relative to RPE (1-10) | | | | | |
|-----------|----------|------------|------|-----------------------------|-----|------|-----|-----|------|------|------|-----|-----|-------|------|-----|-----|------------------------|------|------|------------|------|-----|
| | | % | min | Time (%) | | | | | | | | | | | | | | Time (%) | | | Time (min) | | |
| | | | | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | >9 | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | >9 |
| Today | Today | 17.9 | 44.8 | 24.1 | 14 | 16.7 | 15 | 12 | 8.2 | 5.2 | 2 | 1.3 | 1.6 | 60.25 | 35.5 | 42 | 38 | 28.75 | 20.5 | 13 | 5 | 3.25 | 4 |
| | Tour Avs | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Min | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Max | | | | | | | | | | | | | | | | | | | | | | |
| Yesterday | Today | 21.5 | 53.8 | 28 | 13 | 16.1 | 15 | 11 | 7.38 | 4.65 | 2.35 | 1.3 | 1.5 | 70 | 32.5 | 40 | 37 | 27.75 | 18.5 | 11.6 | 5.88 | 3.2 | 3.8 |
| | Tour Avs | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Min | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Max | | | | | | | | | | | | | | | | | | | | | | |

Peak Power Output:

| | | Average Power (watts) | | | | | | | | Distance from Start (km) | | | | | | | | # of Surges > than a W/kg of: | | | Hydration & Energy Status | | | | | |
|-------|----------|-----------------------|-----|-----|-----|-----|-----|------|----|--------------------------|-----|-----|-----|-----|-----|------|----|----------------------------------|-----|------|---------------------------|-------------|----------|--------|-------|-------------|
| | | Sec | | Min | | | | Hour | | Sec | | Min | | | | Hour | | | | | Weight (kg) | | | Bottle | Sweat | |
| | | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | > 6 | > 8 | > 10 | Pre | Post | Δ | % Δ | Count | Loss (l) |
| Rider | Today | 957 | 853 | 628 | 496 | 369 | 315 | 252 | NA | 181 | 181 | 6.4 | 6.1 | 3.7 | 175 | 158 | NA | 316 | 99 | 41 | 70.3 | 70 | 0.3 | 0.4267 | 10 | 5.1 |
| | Tour Avs | | | | | | | | | | | | | | | | | | | | Est | Sweat Rates | | % | | |
| | Tour Min | | | | | | | | | | | | | | | | | | | | Loss (l) | l/hr | l/mjoule | GME | Kcals | |
| | Tour Max | | | | | | | | | | | | | | | | | | | | | 4.15 | 1.22 | 1.66 | 24 | 3060.797898 |
| | | | | | | | | | | | | | | | | | | | | | Race Food Eaten: | | | | | |
| Rider | Today | 948 | 612 | 494 | 362 | 320 | 244 | 214 | NA | 151 | 95 | 95 | 93 | 132 | 120 | 59 | NA | 289 | 113 | 39 | | | | | | |
| | Tour Avs | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Min | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Max | | | | | | | | | | | | | | | | | | | | | | | | | |

Climbs and Special Features:

| Climb/Feature: | Start (km) | Top (km) | Total Dist | % Grade | Calc Grade | Net Dist | Top Elev | Total Gain | Time | | Speed km/hr | VAM m/hr | Estimated Power | | | | Actual | | % Diff | |
|------------------------------|---------------|-------------|---------------|------------|---------------|-------------|-------------|---------------|------|-----|----------------|-------------|-----------------|------|------|-------|--------|-----|-------------|--|
| | | | | | | | | | Min | Sec | | | Roll | Acro | Grav | Total | Power | Cad | | |
| | | | | | | | | | | | | | | | | | | | | |
| 1. Cote de Heiligenstein (4) | 100.4 | 101.5 | 1.1 | 4.1 | 4.10 | 244.9 | 290 | 45.1 | 2 | 11 | 30.22901 | 1239 | 32 | 110 | 261 | 402 | 361 | 88 | 10.27199917 | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |



Saris Cycling Team

Argyle & Co. Ltd. (UK) 01463 244704 (UK) 01463 244705 (UK) 01463 244706 (UK) 01463 244707 (UK)



Page 2: Overhill to Each-Sar-Algona, 228.5 km

Monday, July 2nd

Floyd Landis

Temp: 88 °F / 31 °C Humidity: 45 % Heat Index: 89.4 °F / 31.86 °C

Stage Results: 2:55.5 hrs 20.8 km/hr 25.2 mph

GC Results: 2:20.5 hrs 24.4 km/hr 26.3 mph

Place: 30 Time: 5 hrs 36 min 14 sec Gap: 0 min 0 sec

Place: 11 Time: 9 hrs 54 min 40 sec Gap: 0 hrs 0 min 21 sec

Race Notes: Today was really similar to yesterday. Floyd felt it was really easy, except for the fact that it felt a lot hotter today despite the temperature not actually being that different. Lots of solar radiation. Seemed like more work on hills. Floyd says he drank 20 bottles. Forgot weights. No GPS.

Power, RPE, Heart Rate, Work, RPE vs. Power, HR vs. Power

| | | Power when Moving | | Power when Pedaling | | Strain | | Work or Stress in Kjoules From: | | | RPE | | HR | |
|-------|----------|-------------------|----------|---------------------|----------|--------|-----|---------------------------------|-------|------|------|--|------|--|
| | | Watts | Watts/kg | Watts | Watts/kg | RPE | HR | Power | RPE | HR | Pwr | | Pwr | |
| | | | | | | | | | | | | | | |
| Rider | Today | 195 | 2.80 | 256 | 3.67 | 3.5 | NA | 3,934 | 4,187 | NA | 1.06 | | NA | |
| | Tour Avs | 203 | 2.91 | 256 | 3.69 | 3.5 | NA | 3,520 | 3,679 | NA | 1.05 | | NA | |
| | Tour Min | 195 | 2.8 | 250 | 3.6 | 3.5 | NA | 3,075 | 3,113 | NA | 1.01 | | NA | |
| | Tour Max | 205 | 2.95 | 257 | 3.7 | 3.5 | NA | 3,934 | 4,187 | NA | 1.06 | | NA | |
| Bike | Today | 194 | 2.78 | 261 | 3.7 | 3.5 | 120 | 3,904 | NA | 4035 | | | 1.03 | |
| | Tour Avs | 198 | 2.8 | 260 | 3.65 | 3.95 | 122 | 3,571 | NA | 3618 | | | 1.01 | |
| | Tour Min | 186 | 2.78 | 249 | 3.6 | 3.5 | 120 | 2,925 | NA | 3200 | | | 1.09 | |
| | Tour Max | 205 | 2.83 | 273 | 3.7 | 4.4 | 124 | 3,994 | NA | 4035 | | | 1.01 | |

Power Distribution

| | | Zero Watts | | Per Kilogram of Body Weight | | | | | | | | | | | | | | | | Relative to RPE (1-10) | | | | | | | | | |
|-------|----------|------------|------|-----------------------------|-----|------|-----|-----|-----|------|------|-----|------|------------|-----|-----|-----|-------|------|------------------------|------|-----|------|----------|------|------|------------|-------|---------|
| | | | | Time (%) | | | | | | | | | | Time (min) | | | | | | | | | | Time (%) | | | Time (min) | | |
| | | | | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | 9-10 | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | 9-10 | <H | H | >H | <H | H | >H |
| Rider | Today | 23.9 | 80.4 | 30 | 13 | 12.8 | 13 | 12 | 8.8 | 6 | 3 | 1.1 | 1 | 100.87 | 43 | 43 | 43 | 39.34 | 29.6 | 20.2 | 10.1 | 3.7 | 3.4 | 68 | 21 | 11 | 230 | 68.88 | 36.9856 |
| | Tour Avs | 20.9 | 64.0 | 27.1 | 14 | 14.8 | 14 | 12 | 8.5 | 5.6 | 2.5 | 1.2 | 1.2 | 82 | 39 | 42 | 40 | 34 | 25 | 17 | 7.8 | 3.5 | 3.6 | 69.3 | 20.1 | 10.6 | 203.7 | 59.65 | 31.7 |
| | Tour Min | 17.9 | 45.0 | 24.1 | 13 | 12.8 | 13 | 12 | 8.2 | 5.2 | 2 | 1.1 | 1 | 60 | 36 | 42 | 38 | 29 | 21 | 13 | 5 | 3.3 | 3.4 | 68.4 | 19.7 | 10.1 | 175.5 | 49.25 | 25.25 |
| | Tour Max | 23.9 | 80.4 | 30 | 14 | 16.7 | 15 | 12 | 8.8 | 6.3 | 3 | 1.3 | 1.6 | 101 | 43 | 43 | 43 | 39 | 40 | 20 | 10 | 3.7 | 4 | 70.2 | 20.5 | 11.1 | 229.8 | 68.88 | 37.3 |
| Bike | Today | 25.9 | 87.0 | 31.2 | 12 | 13.2 | 13 | 12 | 9 | 5.45 | 2.55 | 1.1 | 1 | 105 | 40 | 44 | 43 | 40 | 30 | 18 | 8.6 | 3.5 | 3.4 | 69.1 | 20.9 | 10.1 | 232 | 70.22 | 33.77 |
| | Tour Avs | 24.1 | 75.0 | 30 | 13 | 14.2 | 13 | 12 | 8.4 | 5.14 | 2.4 | 1.2 | 1.3 | 93 | 38 | 42 | 40 | 36 | 26 | 16 | 7.5 | 3.4 | 3.7 | 70.1 | 19.9 | 9.98 | 213 | 61.76 | 30.5 |
| | Tour Min | 17.9 | 45.0 | 24.1 | 9.9 | 12.8 | 12 | 10 | 6.8 | 4.1 | 1.7 | 0.8 | 0.8 | 60 | 30 | 35 | 32 | 26 | 17 | 10 | 4.3 | 2.5 | 2.7 | 68.4 | 17.1 | 8.5 | 175.5 | 42.75 | 21.25 |
| | Tour Max | 27.4 | 92.0 | 32.6 | 14 | 16.8 | 15 | 12 | 9.3 | 6 | 3 | 1.6 | 2.2 | 110 | 44 | 46 | 47 | 42 | 31 | 20 | 10 | 4 | 5.5 | 73.5 | 21.5 | 11.5 | 235.2 | 72.24 | 37.3 |

Peak Power Output

| | | Average Power (watts) | | | | | | Distance from Start (km) | | | | | | # of surges > than w/kg of: | | | Weight (kg) | | | | | | Bottle | | Sweat | |
|-------|----------|-----------------------|-----|------|-----|-----|------|--------------------------|-----|------|-----|-----|------|-----------------------------|-----|------|-------------|-------------|-----|--------|-------|----------|--------|----------|-------|--|
| | | Sec | Min | Hour | Sec | Min | Hour | Sec | Min | Hour | Sec | Min | Hour | >6 | >8 | >10 | Pre | Post | Δ | % Δ | Count | Loss (l) | Count | Loss (l) | | |
| | | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 5 | 10 | 30 | 1 | 5 | | | | 69.8 | 69.6 | 0.2 | 0.2865 | 15 | 7.4 | | | | |
| | | | | | | | | | | | | | | | | | Est | Sweat Rates | | | % | | | | | |
| Rider | Today | 889 | 528 | 497 | 409 | 380 | 329 | 267 | NA | 168 | 212 | 212 | 219 | 210 | 202 | 181 | NA | 365 | 94 | 22 | | | | | | |
| | Tour Avs | 923 | 578 | 497 | 389 | 348 | 291 | 241 | NA | 175 | 109 | 109 | 111 | 193 | 180 | 97.7 | NA | 339 | 95 | 30 | | | | | | |
| | Tour Min | 889 | 528 | 496 | 369 | 315 | 252 | 214 | NA | 168 | 6.4 | 6.1 | 3.7 | 175 | 158 | 14.3 | NA | 316 | 94 | 22 | | | | | | |
| | Tour Max | 957 | 628 | 497 | 409 | 380 | 329 | 267 | NA | 181 | 212 | 212 | 219 | 210 | 202 | 181 | NA | 365 | 99 | 42 | | | | | | |
| Bike | Today | 919 | 554 | 502 | 386 | 364 | 308 | 263 | NA | 164 | 195 | 186 | 169 | 207 | 199 | 177 | NA | 351 | 101 | 22 | | | | | | |
| | Tour Avs | 918 | 569 | 498 | 378 | 347 | 283 | 237 | NA | 158 | 169 | 162 | 157 | 195 | 183 | 139 | NA | 330 | 107 | 28 | | | | | | |
| | Tour Min | 809 | 528 | 463 | 329 | 304 | 235 | 211 | NA | 85 | 6.4 | 6.7 | 3.7 | 4.2 | 4.9 | 4.9 | NA | 245 | 79 | 16 | | | | | | |
| | Tour Max | 1070 | 642 | 544 | 409 | 380 | 329 | 267 | NA | 182 | 212 | 212 | 219 | 210 | 202 | 181 | NA | 394 | 148 | 50 | | | | | | |

Climbs and Special Features

| Climb/Feature: | Start | Top | Total | % | Calc | Start | Top | Total | Time | | Speed | VAM | Estimated Power | | | | Arrival | | % | |
|-----------------------------|---|---------|-------|-----|-----------|--------|-----|-------|------|-----|----------|------|-----------------|------|------|-------|---------|------|------------|--|
| | (km) | (m) | (m) | (m) | Grade | (km) | (m) | Gain | Min | Sec | km/hr | m/hr | Roll | Aero | Grav | Total | Power | Cost | Diff | |
| | | | | | | | | | | | | | | | | | | | | |
| 1. Col des Pandours (3) | 27.7 | 35.5 | 7.8 | 4.1 | 4.106021 | 342 | 662 | 320 | 18 | 35 | 25.18386 | 1033 | 27 | 63 | 218 | 307 | 280 | 83 | 8.91299132 | |
| 2. Col de Valsberg (3) | 46.4 | 50 | 3.6 | 5.2 | 5.2014665 | 465 | 652 | 187 | 8 | 15 | 26.18182 | 1360 | 28 | 71 | 286 | 385 | 341 | 89 | 11.4506867 | |
| 3. Cote de Kedange (4) | 186.3 | 187.5 | 1.2 | 5.9 | 5.9052696 | 182.26 | 253 | 70.74 | 2 | 28 | 29.18919 | 1721 | 31 | 99 | 362 | 492 | 413 | 90 | 15.9990189 | |
| 4. Cote de Kanfen (4) | 210.6 | 212.5 | 1.9 | 4.5 | 4.5019236 | 239.55 | 325 | 85.45 | 3 | 30 | 32.57143 | 1465 | 34 | 137 | 308 | 480 | 406 | 95 | 15.3734561 | |
| 5. Cote de Vomrange (4) | 213.6 | 215 | 1.4 | 6.5 | 6.5001172 | 319.19 | 410 | 90.81 | 3 | 18 | 26.52632 | 1721 | 28 | 74 | 362 | 464 | 450 | 90 | 3.06668018 | |
| | | | | | | | | | | | | | | | | | | | | |
| Big Surge Late in Race | 217.8 | 218.107 | 0.333 | | | | | | 0 | 22 | 54.49091 | | 57 | 642 | | 699 | 556 | | 20.4495767 | |
| Last 3.5 Kilometers (Spiky) | Two surges over 10 w/kg. Once for 5 sec & once for 4 sec. | | | | | | | | 3 | 40 | | | | | | | 322 | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |

GDC01081



Personal Cycling Team

Address: 461 E. 1st St., CH 4034, Hamburg, MO. Tel: +1 (816) 264-7005 Fax: +1 (816) 264-7001



Le Tour 2006: Daily Analysis Report

Stage 1: Esch-Sur-Alzette to Lulenburg, 216.5 km

Tuesday, July 4th

Floyd Landis

Temp: 95 °F / 35 °C Humidity: 45 % Heat Index: 102 °F / 38.83 °C

Stage Results: 216.5 km 43.6 km/hr 21.2 mph

GC Results: 630.6 km ridden 42.5 km/hr 26.1 mph

Place: 44 Time: 4 hrs 57 min 59 sec Gap: 0 min 5 sec

Place: 7 Time: 14 hrs 52 min 39 sec Gap: 0 hrs 0 min 16 sec

Race Notes: Today was super hot. John says the temp from the car read 37 °C. It was also really up and down today with much harder efforts for all of the riders. We rode through Bastogne and into Liege today as well. Historic stuff.

Power, RPE, Heart Rate, Work, RPE vs. Power, HR vs. Power

| | | Power when Moving | | Power when Pedaling | | Strain | | Work or Stress in Kjoules From: | | | RPE | | HR | |
|---------|----------|-------------------|----------|---------------------|----------|--------|----|---------------------------------|-------|----|------|--|-----|--|
| | | Watts | Watts/kg | Watts | Watts/kg | RPE | HR | Power | RPE | HR | Pwr | | Pwr | |
| Stage 1 | Today | 222 | 3.18 | 276 | 3.96 | 4 | NA | 3,969 | 4,066 | NA | 1.02 | | NA | |
| | Tour Avs | 205 | 2.95 | 259 | 3.7 | 3.73 | NA | 3,668 | 3,806 | NA | 1.03 | | NA | |
| | Tour Min | 195 | 2.81 | 250 | 3.6 | 3.5 | NA | 3075 | 3113 | NA | 1.01 | | NA | |
| | Tour Max | 222 | 3.19 | 276 | 3.97 | 4 | NA | 3969 | 4187 | NA | 1.06 | | NA | |
| GC | Today | 214 | 3.07 | 275 | 3.9 | 4.3 | NA | 3856 | NA | NA | NA | | NA | |
| | Tour Avs | 201 | 2.88 | 262 | 3.8 | 3.44 | NA | 3614 | NA | NA | NA | | NA | |
| | Tour Min | 186 | 2.73 | 249 | 3.5 | 3 | NA | 2925 | NA | NA | NA | | NA | |
| | Tour Max | 222 | 3.19 | 284 | 4 | 4.5 | NA | 3994 | NA | NA | NA | | NA | |

Power Distribution:

| | | | Zero Watts | | Per Kilogram of Body Weight | | | | | | | | | | | | | | | | | | Relative to RPE (1-10) | | | | | | |
|-------|----------|------|------------|------|-----------------------------|------|-----|-----|------|------|------|-----|-----|-------|------------|-----|-----|-------|------|------|------|------|------------------------|------|------|------------|-------|-------|-------|
| | | | | | Time (%) | | | | | | | | | | Time (min) | | | | | | | | Time (%) | | | Time (min) | | | |
| | | | % | min | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | >9 | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | >9 | <H | H | >H | <H | H |
| Today | Today | 19.6 | 58.4 | 24.3 | 11 | 13.2 | 14 | 13 | 11.6 | 6.8 | 2.9 | 1.4 | 1.6 | 72.41 | 33.1 | 39 | 42 | 39.04 | 34.6 | 20.3 | 8.64 | 4.17 | 4.8 | 63 | 25 | 13 | 186 | 73 | 38 |
| | Tour Avs | 20.5 | 62.0 | 26.1 | 13 | 14.2 | 14 | 12 | 9.5 | 6 | 2.6 | 1.3 | 1.4 | 79 | 37 | 41 | 41 | 36 | 28 | 18 | 8.1 | 3.7 | 4 | 67 | 21.6 | 11.3 | 197.8 | 64.23 | 33.71 |
| | Tour Min | 17.9 | 45.0 | 24.1 | 11 | 12.8 | 13 | 12 | 8.2 | 5.2 | 2 | 1.1 | 1 | 60 | 33 | 39 | 38 | 29 | 21 | 13 | 5 | 3.3 | 3.4 | 62.6 | 19.7 | 10.1 | 175.5 | 49.25 | 25.25 |
| | Tour Max | 23.9 | 80.0 | 30 | 14 | 16.7 | 15 | 13 | 11.6 | 6.8 | 3 | 1.4 | 1.6 | 101 | 43 | 43 | 43 | 39 | 34 | 20 | 10 | 4.2 | 4.8 | 70.2 | 24.7 | 12.7 | 229.8 | 73.36 | 37.72 |
| GC | Today | 22.1 | 66.0 | 27 | 10 | 13.2 | 13 | 13 | 11.1 | 6.32 | 2.78 | 1.2 | 1.3 | 81 | 31 | 40 | 40 | 39 | 33 | 19 | 8.3 | 3.7 | 4 | 64.1 | 24.3 | 11.7 | 192.1 | 72.77 | 34.95 |
| | Tour Avs | 23 | 69.0 | 28.5 | 12 | 14.1 | 14 | 12 | 9.38 | 5.55 | 2.62 | 1.2 | 1.3 | 85 | 34 | 41 | 40 | 36 | 28 | 17 | 7.8 | 3.5 | 3.7 | 67.8 | 21.5 | 10.7 | 200.5 | 64.38 | 31.7 |
| | Tour Min | 17.9 | 45.0 | 24.1 | 9.3 | 12.7 | 12 | 10 | 6.8 | 4.1 | 1.7 | 0.8 | 0.8 | 60 | 28 | 35 | 32 | 26 | 17 | 10 | 4.3 | 2.5 | 2.7 | 62.5 | 17.1 | 8.5 | 175.5 | 42.75 | 21.25 |
| | Tour Max | 27.4 | 92.0 | 32.6 | 14 | 16.8 | 15 | 14 | 11.6 | 6.8 | 3.3 | 1.7 | 2.2 | 110 | 44 | 46 | 47 | 42 | 35 | 20 | 10 | 5.1 | 5.5 | 73.5 | 24.8 | 12.8 | 235.2 | 74.15 | 38.14 |

Peak Power Output:

| | | Average Power (watts) | | | | | | | | Distance from Start (km) | | | | | | | | # of surges > than w/kg of: | | | Hydration & Energy Status: | | | | | |
|---------|----------|-----------------------|-----|-----|-----|-----|-----|-----|----|--------------------------|-----|-----|-----|-----|-----|-----|----|-----------------------------|-----|------|----------------------------|------|------|--------|--------------|----------------|
| | | Sec | | | | | | | | Min | | | | | | | | Weight (kg) | | | Pre Post Δ % Δ | | | | | |
| | | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | > 6 | > 8 | > 10 | Pre | Post | Δ | % Δ | Bottle Count | Sweat Loss (l) |
| Stage 1 | Today | 972 | 713 | 674 | 406 | 352 | 289 | 266 | NA | 193 | 213 | 213 | 127 | 125 | 192 | 167 | NA | 390 | 110 | 41 | 69.9 | 69.7 | 0.2 | 0.2861 | 12 | 5.96 |
| | Tour Avs | 939 | 623 | 556 | 395 | 349 | 290 | 249 | NA | 181 | 144 | 144 | 117 | 170 | 184 | 121 | NA | 356 | 100 | 34 | Est | | | | | |
| | Tour Min | 889 | 528 | 496 | 369 | 315 | 252 | 214 | NA | 168 | 6.4 | 6.1 | 3.7 | 125 | 158 | 143 | NA | 316 | 94 | 22 | Loss (l) | | | | | |
| | Tour Max | 972 | 713 | 674 | 409 | 380 | 329 | 267 | NA | 193 | 213 | 213 | 219 | 210 | 202 | 181 | NA | 390 | 110 | 42 | 5.36 | 1.20 | 1.50 | 24 | 3950.806259 | |
| GC | Today | 1001 | 624 | 556 | 410 | 352 | 286 | 263 | NA | 152 | 176 | 132 | 139 | 121 | 141 | 155 | NA | 357 | 98 | 32 | Race Food Eaten: | | | | | |
| | Tour Avs | 957 | 596 | 518 | 386 | 344 | 279 | 245 | NA | 157 | 154 | 136 | 134 | 145 | 148 | 130 | NA | 332 | 100 | 29 | | | | | | |
| | Tour Min | 809 | 528 | 463 | 329 | 304 | 235 | 211 | NA | 16 | 6.4 | 6.1 | 3.7 | 4.2 | 4.9 | 4.9 | NA | 245 | 76 | 16 | | | | | | |
| | Tour Max | 1070 | 713 | 674 | 434 | 380 | 329 | 280 | NA | 199 | 213 | 213 | 219 | 210 | 202 | 181 | NA | 394 | 148 | 50 | | | | | | |

Climbs and Special Features:

| Climb/Feature: | Start (km) | Top (km) | Elev (m) | % Grade | Calc Grade | Start Elev | Top Elev | Total Gain | Total | | Speed km/hr | VAM m/hr | Estimated Power | | | | Actual | | % Diff | |
|------------------------------|---------------|-------------|-------------|------------|---------------|---------------|-------------|---------------|-------|-------|----------------|-------------|-----------------|------|------|-------|--------|-----|------------|--|
| | | | | | | | | | Mile | Sec | | | Roll | Aero | Grav | Total | Power | Cal | | |
| | | | | | | | | | | | | | | | | | | | | |
| 1. Cote de La Haute-Leve (3) | 127.4 | 131 | 3.6 | 5.5 | 5.5083377 | 304 | 502 | 198 | 8 | 12 | 26.34146 | 1449 | 28 | 72 | 305 | 405 | 380 | 89 | 6.23487282 | |
| 2. Cote de Oneux (3) | 151.8 | 155 | 3.2 | 5.1 | 5.1003711 | 164 | 327 | 163 | 7 | 27 | 25.77181 | 1313 | 27 | 68 | 276 | 371 | 360 | 87 | 3.07585632 | |
| 3. Cote de Petit (4) | 163.8 | 165.5 | 1.7 | 4.7 | 4.6992979 | 190.2 | 270 | 79.8 | 3 | 21 | 30.44776 | 1429 | 32 | 112 | 301 | 445 | 372 | 92 | 16.3915394 | |
| 4. Cote de Loorberg (4) | 187.7 | 189 | 1.3 | 5.8 | 5.802049 | 140.7 | 216 | 75.3 | 2 | 39 | 29.43396 | 1705 | 31 | 101 | 359 | 491 | 418 | 95 | 14.8839138 | |
| 5. Cote de Trintelem (4) KE | 199.3 | 201 | 1.7 | 5.1 | 5.1007402 | 108.4 | 195 | 86.6 | 3 | 31.68 | 28.91156 | 1473 | 30 | 96 | 310 | 436 | 436 | 92 | 0.08705756 | |
| 5. Le Cauberg (3) | 213.7 | 214.5 | 0.80 | 7.3 | 7.3006284 | 76.75 | 135 | 58.25 | 1 | 35 | 30.31579 | 2207 | 32 | 110 | 465 | 607 | 552 | 95 | 9.08879463 | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |













Rural Cycling Team

At: Cycling AC (Eckst) 1C16634 (Humboldt) Tel: 1-410-254-7005 Fax: 1-410-254-7011



Le Tour 2006: Daily Analysis Report

Stage 11: Tarbes au Val d'Aspin (Pla-de-Beret) 206.5 km

Thursday, July 13th

Floyd Landis

Temp: 71 °F / 22 °C Humidity: 60 % Heat Index: 75.9 °F / 24.41 °C

Stage Results: 206.5 km 35.2 km/hr 21.0 mph

ACC Results: 1:29:00 American km/hr mph

Place: 3 Time: 6 hrs 6 min 25 sec Gap: 0 min 0 sec

Place: 1 Time: 49 hrs 18 min 7 sec Gap: 0 hrs 0 min 0 sec

Race Notes: Floyd rode brilliantly today. Looked really at ease and patient. Finished third with Menchov and Levi. In Yellow. Amazing. Power profile looked good. Well within Floyd's limits. Best 30 s, 1 min, 5 min, and 10 min on Col de Portillon. Best 30 min on Beret.

Power, RPE, Heart Rate, Work, RPE vs. Power, HR vs. Power

| | | Power when Moving | | Power when Pedaling | | Strain | | Work or Stress in KJoules From: | | | RPE | HR |
|-------|----------|-------------------|----------|---------------------|----------|----------|----|---------------------------------|--------------|----|------|-----|
| | | Watts | Watts/kg | Watts | Watts/kg | RPE | HR | Power | RPE | HR | Pwr | Pwr |
| Today | | 267 | 3.94 | 314 | 4.52 | 7 | NA | 5,870 | 7,619 | NA | 1.30 | NA |
| | Tour Avs | 220 | 3.17 | 271 | 3.9 | 4.33 | NA | 3,927 | 4,327 | NA | 1.08 | NA |
| | Tour Min | 195 | 2.81 | 234 | 3.42 | 3.5 | NA | 2,624 | 2,682 | NA | 1.01 | NA |
| | Tour Max | 267 | 2.81 | 314 | 4.52 | 7 | NA | 5,870 | 7,619 | NA | 1.30 | NA |
| 2006 | | | | | | | NA | | | NA | | NA |
| | Tour Avs | 223 | 3.28 | 268 | 3.94 | 6.3 | NA | 3,911 | 5,348 | NA | 1.34 | NA |
| | Tour Min | 164 (S21) | 2.41 | 214 | 3.15 | 4 | NA | 2,174 (S21) | 2,651 (S21) | NA | 0.88 | NA |
| | Tour Max | 285 (S11) | 4.19 | 314 | 4.62 | 10 (S15) | NA | 5,620 (S15) | 11,286 (S15) | NA | 2.01 | NA |

Power Distribution:

| | | Zero Watts | | Per Kilogram of Body Weight | | | | | | | | | | | | | | | | | | Relative to RPE (1-10) | | | | | | | |
|-------|-------|------------|------|-----------------------------|------|-----|-----|------|------|------|-----|-----|-----|------------|-----|-----|-----|-----|-----|-----|-----|------------------------|-----|------|------------|-------|-------|-------|----|
| | | | | Time (%) | | | | | | | | | | Time (min) | | | | | | | | Time (%) | | | Time (min) | | | | |
| | | | | | | | | | | | | | | | | | | | | | | <H | H | >H | <H | H | >H | | |
| | | % | min | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | 9-9 | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | 9-9 | <H | H | >H | <H | H | >H |
| Today | 15 | 55.0 | 17.2 | 5.5 | 7.9 | 12 | 24 | 21.3 | 8 | 2.7 | 1 | 0.6 | 63 | 20 | 29 | 42 | 89 | 78 | 29 | 10 | 4 | 2 | 42 | 46 | 12 | 154.5 | 166.5 | 45 | |
| | 18.4 | 55.0 | 23.4 | 12 | 13.8 | 14 | 14 | 11.3 | 6.38 | 2.88 | 1.4 | 1.3 | 69 | 35 | 39 | 40 | 42 | 35 | 19 | 8.4 | 3.9 | 3.5 | 63 | 24.9 | 12 | 181.9 | 77.81 | 35.8 | |
| | 13.4 | 29.0 | 17.2 | 5.5 | 7.9 | 10 | 9.8 | 6.9 | 4.5 | 2 | 1 | 0.6 | 44 | 20 | 29 | 31 | 21 | 15 | 9.7 | 5 | 3 | 2.2 | 42 | 16.7 | 9.4 | 154.5 | 35.91 | 22.15 | |
| | 23.9 | 80.0 | 30 | 19 | 19.3 | 15 | 24 | 21.3 | 9.4 | 3.6 | 1.8 | 1.7 | 101 | 52 | 50 | 45 | 89 | 78 | 29 | 10 | 5 | 5 | 73 | 43.5 | 16 | 229.8 | 166.5 | 47.95 | |
| 2006 | Today | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 15 | 41.2 | 20.4 | 11 | 12.4 | 14 | 16 | 14 | 7.22 | 2.91 | 1.2 | 1.1 | 55 | 29 | 34 | 38 | 45 | 40 | 20 | 8 | 3 | 3 | | | | | | | |
| | 26.0 | | 12.6 | 5.7 | 6.9 | 11 | 9.1 | 6.8 | 3.7 | 1.7 | 0.9 | 0.7 | 37 | 16 | 19 | 26 | 20 | 15 | 8 | 4 | 2 | 2 | | | | | | | |
| | 84.0 | | 33.8 | 16 | 16.8 | 17 | 23 | 27.9 | 10.3 | 4.7 | 1.7 | 1.7 | 104 | 42 | 46 | 48 | 73 | 85 | 31 | 14 | 5 | 5 | | | | | | | |

Peak Power Output:

| Average Power (watts) | | | | | | | | | | Distance from Start (km) | | | | | | | | | | # of surges > than w/kg of: | | | Weight (kg) | | | | Bottle Count | Sweat Loss (l) |
|-----------------------|----------|-----|-----|-----|-----|-----|-----|------|-----|--------------------------|-----|-----|-----|------|-----|----|---|-----|-----|-----------------------------|------------------|-------------|-------------|--------|-------------|------|--------------|----------------|
| | | Sec | | Min | | | | Hour | | Sec | | Min | | Hour | | | | | Pre | Post | Δ | % Δ | | | | | | |
| | | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | > 6 | > 8 | > 10 | 70 | 69 | 1 | 1.4286 | 20 | 10.6 | | |
| Today | | 861 | 568 | 529 | 439 | 406 | 377 | 332 | n | 203 | 153 | 153 | 153 | 153 | 190 | 55 | n | 487 | 91 | 22 | Est | Sweat Rates | | | % | | | |
| | Tour Avs | 900 | 627 | 535 | 417 | 359 | 306 | 278 | n | | | | | | | | n | 386 | 105 | 30 | Loss (l) | l/hr | l/mjoule | GME | Kcals | | | |
| | Tour Min | 825 | 528 | 494 | 369 | 315 | 252 | 214 | n | | | | | | | | n | 305 | 89 | 22 | 7.93 | 1.74 | 1.81 | 24 | 5842.888995 | | | |
| | Tour Max | 972 | 912 | 674 | 456 | 406 | 377 | 346 | n | | | | | | | | n | 487 | 141 | 42 | Race Food Eaten: | | | | | | | |
| Today | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Avs | 841 | 555 | 479 | 400 | 360 | 314 | 283 | 255 | | | | | | | | | | | | | | | | | | | |
| | Tour Min | 731 | 465 | 403 | 308 | 283 | 249 | 231 | 193 | | | | | | | | | | | | | | | | | | | |
| | Tour Max | 965 | 645 | 555 | 478 | 435 | 386 | 359 | 315 | | | | | | | | | | | | | | | | | | | |

Climbs and Special Features:

| Climbs and Special Features: | | Total Elevation Gain | | | | 9000 m | | | 29520 ft | | Cl. 6.005 h | | 0.18 | | Bike Wt 7 | | Wt 69.5 | | 76.5 | |
|------------------------------|------------|----------------------|-----------|---------|------------|------------|----------|------------|----------|-------|-------------|----------|-----------------|------|-----------|-------|---------|------|-------------|--|
| Climb/Feature: | Start (km) | Top (km) | Dist (km) | % Grade | Calc Grade | Start Elev | Top Elev | Total Gain | Time | | Speed km/hr | VAM m/hr | Estimated Power | | | | Actual | | % Diff | |
| | | | | | | | | | Min | Sec | | | Roll | Aero | Grav | Total | Power | %kg | | |
| 1. Col du Tourmalet | 56.7 | 75 | 18.3 | 7.7 | 7.7003241 | 710 | 2115 | 1405 | 58 | 21.5 | 18.81479 | 1445 | 20 | 26 | 304 | 330 | 334 | 4.81 | 4.6756785 | |
| 2. Col d'Aspin | 92.6 | 105 | 12.4 | 5.1 | 5.1001687 | 857.4 | 1489 | 631.6 | 30 | 15.66 | 24.5861 | 1252 | 26 | 59 | 264 | 345 | 324 | 4.66 | 7.031325809 | |
| 3. Cote de Peyresourde | 126.3 | 136 | 9.7 | 6.8 | 6.8002048 | 910.9 | 1569 | 658.1 | 28 | 17.3 | 20.45335 | 1388 | 22 | 34 | 292 | 348 | 336 | 4.83 | 3.352099624 | |
| 4. Col du Portillon | 153 | 161 | 8 | 7.9 | 7.8995328 | 663 | 1293 | 630 | 23 | 18.84 | 20.15621 | 1587 | 21 | 32 | 334 | 385 | 386 | 5.55 | 0.494918851 | |
| 5. Puerto de Beret | 191.5 | 204.5 | 13 | 5.5 | 5.5006104 | 1146 | 1860 | 714 | 28 | 18 | 27.56184 | 1514 | 29 | 83 | 319 | 431 | 381 | 5.48 | 11.55709287 | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | |
| | | | | | </ | | | | | | | | | | | | | | | |





Patrik Cycling Team

Any cycling A6-1, Elsona, D68634, Montclair, NJ. Tel: +1 (908) 264-7005, Fax: +1 (908) 264-7011



Le Tour 2006: Daily Analysis Report

Stage 12: Bagnols-Montclair, 230 km

Saturday, July 15th

Floyd Landis

Temp: 95 °F / 35 °C Humidity: 60 % Heat Index: 113 °F / 45.05 °C

Stage Results: 230.0 km, 38.9 km/hr, 24.1 mph

GC Results: 1,440.0 km ridden, 40.8 km/hr, 25.3 mph

Place: 29 Time: 5 hrs 54 min 33 sec Gap: 29 min 57 sec

Place: 2 Time: 59 hrs 52 min 3 sec Gap: 0 hrs 1 min 2 sec

Race Notes: It was so freaking hot today. Way too hot to do anything except nothing. Why was it so hot. Peirero and Voight and Chaval, and

some other guy got away and built a 30 minute gap. Floyd gives up yellow. Super easy. That's a good thing. Time to get some rest the next two days.

Power, RPE, Heart Rate, Work, RPE vs. Power, HR vs. Power

| | Power when Moving | | Power when Pedaling | | Strain | | Work or Stress in Kjoules From: | | | RPE | HR |
|----------|-------------------|----------|---------------------|----------|----------|----|---------------------------------|--------------|----|------|-----|
| | Watts | Watts/kg | Watts | Watts/kg | RPE | HR | Power | RPE | HR | Pwr | Pwr |
| Today | 178 | 2.56 | 217 | 3.12 | 5 | NA | 3,787 | 5,683 | NA | 1.50 | NA |
| Tour Avs | 218 | 3.14 | 266 | 3.84 | 4.5 | NA | 3,926 | 4,528 | NA | 1.13 | NA |
| Tour Min | 178 | 2.56 | 217 | 3.12 | 3.5 | NA | 2,624 | 2,682 | NA | 1.01 | NA |
| Tour Max | 267 | 3.84 | 314 | 4.52 | 7 | NA | 5,870 | 7,619 | NA | 1.50 | NA |
| Today | | | | | | NA | | | NA | | NA |
| Tour Avs | 223 | 3.28 | 268 | 3.94 | 6.3 | NA | 3,911 | 5,348 | NA | 1.34 | NA |
| Tour Min | 164 (S21) | 2.41 | 214 | 3.15 | 4 | NA | 2,174 (S21) | 2,651 (S21) | NA | 0.88 | NA |
| Tour Max | 285 (S11) | 4.19 | 314 | 4.62 | 10 (S15) | NA | 5,620 (S15) | 11,286 (S15) | NA | 2.01 | NA |

Power Distribution

| | | Zero Watts | | Per Kilogram of Body Weight | | | | | | | | | | | | | | | | | | Relative to RPE (1-10) | | | | | | | |
|-------|----------|------------|------|-----------------------------|-----|------|-----|-----|------|------|------|-----|------------|-----|-----|-----|-----|-----|-----|-----|-----|------------------------|-----|----|------------|-----|-------|-------|-------|
| | | | | Time (%) | | | | | | | | | Time (min) | | | | | | | | | Time (%) | | | Time (min) | | | | |
| | | % | min | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | >9 | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | >9 | <H | H | >H | <H | H | >H |
| Rider | Today | 17.8 | 63.0 | 24.4 | 16 | 19.3 | 18 | 12 | 5.4 | 2.4 | 1.1 | 0.4 | 0.6 | 86 | 57 | 68 | 64 | 42 | 19 | 8 | 4 | 1 | 2 | 78 | 17 | 4 | 276.5 | 61.6 | 15.9 |
| | Tour Avs | 18 | 55.0 | 23.1 | 13 | 14.5 | 14 | 14 | 10.8 | 6.09 | 2.81 | 1.3 | 1.2 | 69 | 36 | 42 | 42 | 43 | 34 | 18 | 8.2 | 3.7 | 3.5 | 64 | 24.5 | 11 | 189.9 | 76.05 | 33.51 |
| | Tour Min | 13.4 | 29.0 | 17.2 | 5.5 | 7.9 | 10 | 9.8 | 5.4 | 2.4 | 1.1 | 0.4 | 0.6 | 44 | 20 | 29 | 31 | 21 | 15 | 8.5 | 3.9 | 1.4 | 2.1 | 42 | 16.7 | 4.5 | 154.5 | 35.91 | 15.93 |
| | Tour Max | 23.9 | 80.0 | 30 | 19 | 19.3 | 18 | 24 | 21.3 | 9.4 | 3.8 | 1.7 | 1.7 | 101 | 57 | 68 | 64 | 89 | 78 | 29 | 11 | 5 | 5 | 78 | 45.5 | 16 | 276.5 | 166.5 | 47.95 |
| Team | Today | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Avs | 15 | 41.2 | 20.4 | 11 | 12.4 | 14 | 16 | 14 | 7.22 | 2.91 | 1.2 | 1.1 | 55 | 29 | 34 | 38 | 45 | 40 | 20 | 8 | 3 | 3 | | | | | | |
| | Tour Min | 26.0 | | 12.6 | 5.7 | 6.9 | 11 | 9.1 | 6.8 | 3.7 | 1.7 | 0.9 | 0.7 | 37 | 16 | 19 | 26 | 20 | 15 | 8 | 4 | 2 | 2 | | | | | | |
| | Tour Max | 84.0 | | 33.8 | 16 | 16.8 | 17 | 23 | 27.9 | 10.3 | 4.7 | 1.7 | 1.7 | 104 | 42 | 46 | 48 | 73 | 85 | 31 | 14 | 5 | 5 | | | | | | |

Peak Power Output

| Average Power (watts) | | | | | | | | | | Distance from Start (km) | | | | | | | | # of surges > than w/kg of: | | | Weight (kg) | | | | Bottle | Sweat |
|-----------------------|----------|-----|-----|-----|-----|-----|-----|------|-----|--------------------------|-----|-----|-----|-----|-----|------|---|--------------------------------|-----|----|------------------|-------------|----------|-----|-------------|----------|
| | | Sec | | Min | | | | Hour | | Sec | | Min | | | | Hour | | | | | Pre | Post | Δ | % Δ | Count | Lost (l) |
| | | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | 69 | 67 | 2 | 2.8986 | 20 | 11.6 | | | |
| Radar | Today | 833 | 569 | 471 | 340 | 296 | 269 | 223 | n | 181 | 6.4 | 6.1 | 3.7 | 175 | 158 | 14.3 | n | 201 | 49 | 14 | Est | Sweat Rates | | % | | |
| | Tour Avs | 894 | 622 | 529 | 414 | 360 | 305 | 275 | n | | | | | | | | n | 373 | 101 | 29 | Loss (l) | 1/hr | 1/mjoule | GME | Kcals | |
| | Tour Min | 825 | 528 | 471 | 340 | 296 | 252 | 214 | n | | | | | | | | n | 201 | 49 | 14 | 5.12 | 1.96 | 3.06 | 24 | 3769.509476 | |
| | Tour Max | 972 | 912 | 674 | 465 | 426 | 377 | 346 | n | | | | | | | | n | 487 | 141 | 43 | Race Food Eaten: | | | | | |
| Radar | Today | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Avs | 841 | 555 | 479 | 400 | 360 | 314 | 283 | 255 | | | | | | | | | | | | | | | | | |
| | Tour Min | 731 | 465 | 403 | 308 | 283 | 249 | 231 | 193 | | | | | | | | | | | | | | | | | |
| | Tour Max | 965 | 645 | 555 | 478 | 435 | 386 | 359 | 315 | | | | | | | | | | | | | | | | | |

Climbs and Special Features

| Climb/Feature: | Start | Top | Total | % | Calc | Start | Top | Total | Time | | Speed | VAM | Estimated Power | | | | Actual | | % |
|-------------------------------|-------|-------|-------|-----|-----------|--------|-----|--------|------|-------|----------|------|-----------------|------|------|-------|--------|------|------------|
| | (km) | (m) | (m) | (%) | Grade | (km) | (m) | (m) | Min | Sec | km/hr | m/hr | Roll | Aero | Grav | Total | Power | %kg | Diff |
| | | | | | | | | | | | | | | | | | | | |
| 1. Cote de Puechabon | 54.9 | 57.5 | 2.6 | 5.2 | 5.201252 | 154.95 | 290 | 135.05 | 7 | 13 | 21.61663 | 1123 | 23 | 40 | 236 | 299 | 280 | 4.03 | 6.4282440 |
| 2. Col de la Cardonille | 75.3 | 77.5 | 2.2 | 5.5 | 5.5014885 | 209.15 | 330 | 120.85 | 5 | 4 | 26.05263 | 1431 | 27 | 70 | 301 | 399 | 312 | 4.49 | 21.7823362 |
| 3. Cote de l'Arbousset | 118 | 119.5 | 1.5 | 4.5 | 4.5012197 | 162.55 | 230 | 67.45 | 3 | 29 | 25.83732 | 1162 | 27 | 68 | 245 | 340 | 306 | 4.40 | 10.061158 |
| 4. Cote de Saint-Maurice | 194.4 | 195.5 | 1.1 | 4.6 | 4.6003146 | 255.45 | 306 | 50.55 | 2 | 42.34 | 24.36323 | 1120 | 26 | 57 | 236 | 319 | 305 | 4.39 | 4.31048082 |
| 5. Cote de Villeneuve de Berg | 203.1 | 205 | 1.9 | 5.3 | 5.3021742 | 291.4 | 392 | 100.6 | 4 | 48 | 23.75 | 1258 | 25 | 53 | 265 | 343 | 319 | 4.39 | 6.97218274 |
| | | | | | | | | | | | | | | | | | | | |
| * All climbs were cat 4's. | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | |





Physiolog Team

Analysing AG | Eichen | CH-8634 | Hombach | Tel: +41 (0)55 254 70 09 | Fax: +41 (0)55 254 70 11



Le Tour 2006: Daily Analysis Report

Stage: 15: Gap to L'Alpe d'Huez, 137 km

Tuesday, July 18th

Floyd Landis

Temp: 90 °F / 32 °C Humidity: 60 % Heat Index: 99.7 °F / 37.6 °C

Stage Results: 137.0 km 39.2 km/hr 23.7 mph

GC Results: 2,614.0 km 39.2 km/hr 23.7 mph

Place: 4 Time: 4 hrs 53 min 32 sec Gap: 1 min 10 sec

Place: 1 Time: 69 hrs 0 min 5 sec Gap: 0 hrs 0 min 0 sec

Race Notes: Floyd had the broken axle today and the mechanics forgot to switch out the computer with the wheel change. So we lost data.

This report is for the first 100 km and the other is calculated off of the math model.

Power, RPE, Heart Rate, Work, RPE vs. Power, HR vs. Power

| | Power when Moving | | Power when Pedaling | | Strain | | Work or Stress in Kjoules From: | | | RPE | | HR | |
|----------|-------------------|----------|---------------------|----------|----------|----|---------------------------------|--------------|----|------|--|-----|--|
| | Watts | Watts/kg | Watts | Watts/kg | RPE | HR | Power | RPE | HR | Pwr | | Pwr | |
| Today | 270 | 3.94 | 321 | 4.59 | 8 | NA | 4,755 | 6,804 | NA | 1.43 | | NA | |
| Tour Avs | | | | | | NA | | | NA | | | NA | |
| Tour Min | 178 | 2.56 | 217 | 3.12 | 3.5 | NA | 2,624 | 2,682 | NA | 1.01 | | NA | |
| Tour Max | 270 | 3.94 | 321 | 4.59 | 8 | NA | 5,870 | 7,619 | NA | 1.50 | | NA | |
| Today | | | | | | NA | | | NA | | | NA | |
| Tour Avs | 223 | 3.28 | 268 | 3.94 | 6.3 | NA | 3,911 | 5,348 | NA | 1.34 | | NA | |
| Tour Min | 164 (S21) | 2.41 | 214 | 3.15 | 4 | NA | 2,174 (S21) | 2,651 (S21) | NA | 0.88 | | NA | |
| Tour Max | 285 (S11) | 4.19 | 314 | 4.62 | 10 (S15) | NA | 5,620 (S15) | 11,286 (S15) | NA | 2.01 | | NA | |

Power Distribution

| | | Zero Watts | | Per Kilogram of Body Weight | | | | | | | | | | | | | | | | | | Relative to RPE (1-10) | | | | | | | |
|-------|----------|------------|------|-----------------------------|-----|------|-----|-----|------|------|------|-----|------|------------|-----|-----|-----|-----|-----|-----|-----|------------------------|------|----|------------|-----|-------|-------|-------|
| | | | | Time (%) | | | | | | | | | | Time (min) | | | | | | | | Time (%) | | | Time (min) | | | | |
| | | % | min | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | 9-10 | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | 9-10 | <H | H | >H | <H | H | >H |
| Rider | Today | 15.3 | 39.0 | 18.5 | 6.7 | 10 | 15 | 18 | 13.2 | 8.5 | 4.7 | 2.4 | 2.5 | 47 | 17 | 25 | 39 | 46 | 34 | 22 | 12 | 6 | 6 | 51 | 31 | 18 | 128.5 | 79.5 | 46 |
| | Tour Avs | | | 23 | 12 | 14 | 14 | 14 | 11 | 6.28 | 2.95 | 1.4 | 1.3 | 68 | 35 | 41 | 42 | 43 | 34 | 18 | 8.4 | 3.9 | 3.7 | 63 | 25 | 12 | 185.8 | 76.28 | 34.35 |
| | Tour Min | 13.4 | 29.0 | 17.2 | 5.5 | 7.9 | 10 | 9.8 | 5.4 | 2.4 | 1.1 | 0.4 | 0.6 | 44 | 17 | 25 | 31 | 21 | 15 | 8.5 | 3.9 | 1.4 | 2.1 | 42 | 16.7 | 4.5 | 129 | 35.91 | 15.93 |
| | Tour Max | 23.9 | 80.0 | 30 | 19 | 19.3 | 18 | 24 | 21.3 | 9.4 | 5 | 2.4 | 3 | 101 | 57 | 68 | 64 | 89 | 78 | 29 | 12 | 6 | 6 | 78 | 45.5 | 16 | 276.5 | 166.5 | 47.95 |
| Team | Today | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Avs | 15 | 41.2 | 20.4 | 11 | 12.4 | 14 | 16 | 14 | 7.22 | 2.91 | 1.2 | 1.1 | 55 | 29 | 34 | 38 | 45 | 40 | 20 | 8 | 3 | 3 | | | | | | |
| | Tour Min | | 26.0 | 12.6 | 5.7 | 6.9 | 11 | 9.1 | 6.8 | 3.7 | 1.7 | 0.9 | 0.7 | 37 | 16 | 19 | 26 | 20 | 15 | 8 | 4 | 2 | 2 | | | | | | |
| | Tour Max | | 84.0 | 33.8 | 16 | 16.8 | 17 | 23 | 27.9 | 10.3 | 4.7 | 1.7 | 1.7 | 104 | 42 | 46 | 48 | 73 | 85 | 31 | 14 | 5 | 5 | | | | | | |

Peak Power Output

| | | Average Power (watts) | | | | | | | | Distance from Start (km) | | | | | | | | # of surges > than w/kg of: | | | Weight (kg) | | | | Bottle | Sweat | |
|------|----------|-----------------------|-----|-----|-----|-----|-----|------|-----|--------------------------|----|-----|---|----|----|------|-----|--------------------------------|-----|------------------|-------------|----------|-----|-----------|--------|----------|--|
| | | Sec | | Min | | | | Hour | | Sec | | Min | | | | Hour | | | | | Pre | Post | Δ | % Δ | Count | Loss (l) | |
| | | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | > 6 | > 8 | > 10 | 69 | 68 | 1 | 1.4493 | 15 | 8.2 | |
| Ride | Today | | | | | | | | | | | | | | | n | | | | | | | | | | | |
| | Tour Avs | 894 | 618 | 530 | 417 | 364 | 307 | 277 | | | | | | | | n | 381 | 107 | 31 | Est | Sweat Rates | | | % | | | |
| | Tour Min | 825 | 528 | 471 | 340 | 296 | 252 | 214 | | | | | | | | n | 201 | 49 | 14 | Loss (l) | l/hr | l/mjoule | GME | Kcals | | | |
| | Tour Max | 972 | 912 | 674 | 465 | 426 | 377 | 346 | | | | | | | | n | 498 | 188 | 50 | 6.42 | 1.68 | 1.72 | 24 | 4733.0387 | | | |
| Ride | Today | | | | | | | | | | | | | | | | | | | Race Food Eaten: | | | | | | | |
| | Tour Avs | 841 | 555 | 479 | 400 | 360 | 314 | 283 | 255 | | | | | | | | | | | | | | | | | | |
| | Tour Min | 731 | 465 | 403 | 308 | 283 | 249 | 231 | 193 | | | | | | | | | | | | | | | | | | |
| | Tour Max | 965 | 645 | 555 | 478 | 435 | 386 | 359 | 315 | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Climbs and Special Features

| Climb/Feature: | Total Elevation Gain | | | | | | | | | | Cr 0.005 k 0.18 Bike Wt 7 Wt 68.5 75.5 | | | | | | | | | |
|--|----------------------|----------|-----------|---------|------------|-----------|----------|-----------|------------|------------|--|------------|-----------------|------|------|-------|--------|------|-------------|--|
| | Start (km) | Top (km) | Dist (km) | % Grade | Calc Grade | Dist (km) | Top (km) | Dist (km) | Total Gain | Time (min) | Speed (km/hr) | VAM (m/hr) | Estimated Power | | | | Actual | | % Diff | |
| | | | | | | | | | | | | | Roll | Acro | Grav | Total | Power | %/kg | | |
| 1. Col de l'Isard | 71.8 | 86 | 14.2 | 7 | 6.9959308 | 1369 | 2360 | 991 | 40 | 23 | 21.09781 | 1472 | 22 | 37 | 310 | 189 | 342 | 4.92 | 7.437836622 | |
| 2. Col du Lautaret* | 121.9 | 134 | 12.1 | 4.4 | 4.40095 | 1526 | 2058 | 532 | 24 | 30 | 29.63265 | 1303 | 31 | 103 | 274 | 402 | 390 | 5.61 | 4.577309629 | |
| 3. L'Alpe d'Huez | 173.2 | 187 | 13.8 | 7.9 | 7.9013614 | 763 | 1850 | 1087 | 38 | 34 | 21.46932 | 1691 | 23 | 39 | 356 | 418 | 410 | 5.90 | 1.800075839 | |
| Actual time up L'Alpe d'Huez. Time given by official race course timers who timed the climb. | | | | | | | | | | | | | | | | | | | | |
| * Time Estimated | | | | | | | | | | | | | | | | | | | | |



Le Tour 2006

Amateur Cycling Team



Le Tour 2006: Daily Analysis Report

Stage: 13 - Bourg-D'Oisans to La Toussuire, 182 km

Wednesday, July 17th

Floyd Landis

Temp: 90 °F / 32 °C Humidity: 60 % Heat Index: 99.7 °F / 37.6 °C

Stage Results: 5:21.2 hrs 31.5 km/h 19.6 mph

31C Results: 2:29:10 Am riders 40.0 km/hr 24.8 mph

Place: 23 Time: 5 hrs 46 min 8 sec Gap: 10 min 4 sec

Place: 11 Time: 74 hrs 38 min 5 sec Gap: 0 hrs 8 min 8 sec

Race Notes: Bad Day. Floyd bonked on last climb. Didn't eat or drink enough. Missing last 6 km of Toussuire...

Power, RPE, Heart Rate, Watt, RPE vs. Power, HR vs. Power

| | | Power when Moving | | Power when Pedaling | | Strain | | Work or Stress in Kjoules From: | | | RPE | HR |
|-----------|----------|-------------------|----------|---------------------|----------|----------|----|---------------------------------|--------------|----|------|-----|
| | | Watts | Watts/kg | Watts | Watts/kg | RPE | HR | Power | RPE | HR | Pwr | Pwr |
| Today | Today | 259 | 3.73 | 312 | 4.48 | 10 | NA | 5,379 | 8,848 | NA | 1.64 | NA |
| | Tour Avs | | | | | | NA | | | NA | | NA |
| | Tour Min | 178 | 2.56 | 217 | 3.12 | 3.5 | NA | 2,624 | 2,682 | NA | 1.01 | NA |
| | Tour Max | 267 | 3.84 | 314 | 4.52 | 7 | NA | 5,870 | 7,619 | NA | 1.50 | NA |
| Yesterday | Today | | | | | | NA | | | NA | | NA |
| | Tour Avs | 223 | 3.28 | 268 | 3.94 | 6.3 | NA | 3,911 | 5,348 | NA | 1.34 | NA |
| | Tour Min | 164 (S21) | 2.41 | 214 | 3.15 | 4 | NA | 2,174 (S21) | 2,651 (S21) | NA | 0.88 | NA |
| | Tour Max | 285 (S11) | 4.19 | 314 | 4.62 | 10 (S15) | NA | 5,620 (S15) | 11,286 (S15) | NA | 2.01 | NA |

Power Distribution

| | | Zero Watts | | Per Kilogram of Body Weight | | | | | | | | | | | | | | Relative to RPE (1-10) | | | | | |
|-----------|----------|------------|------|-----------------------------|-----|------|-----|-----|------|------|------|-----|-----|-----|-----|-----|-----|------------------------|-----|-----|-----|-----|-----|
| | | % min | min | Time (%) | | | | | | | | | | | | | | Time (min) | | | | | |
| | | | | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | >9 | 0-1 | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 | 8-9 | >9 |
| Today | Today | 16.9 | 58.0 | 20.1 | 4.8 | 5.5 | 12 | 26 | 20.8 | 6.8 | 1.9 | 1.9 | 0 | 70 | 17 | 19 | 42 | 90 | 72 | 24 | 7 | 7 | 0 |
| | Tour Avs | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Min | 13.4 | 29.0 | 17.2 | 5.5 | 7.9 | 10 | 9.8 | 5.4 | 2.4 | 1.1 | 0.4 | 0.6 | 44 | 20 | 29 | 31 | 21 | 15 | 8.5 | 3.9 | 1.4 | 2.1 |
| | Tour Max | 23.9 | 80.0 | 30 | 19 | 19.3 | 18 | 24 | 21.3 | 9.4 | 3.8 | 1.7 | 1.7 | 101 | 57 | 68 | 64 | 89 | 78 | 29 | 11 | 5 | 5 |
| Yesterday | Today | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Avs | 15 | 41.2 | 20.4 | 11 | 12.4 | 14 | 16 | 14 | 7.22 | 2.91 | 1.2 | 1.1 | 55 | 29 | 34 | 38 | 45 | 40 | 20 | 8 | 3 | 3 |
| | Tour Min | 26.0 | | 12.6 | 5.7 | 6.9 | 11 | 9.1 | 6.8 | 3.7 | 1.7 | 0.9 | 0.7 | 37 | 16 | 19 | 26 | 20 | 15 | 8 | 4 | 2 | 2 |
| | Tour Max | 84.0 | | 33.8 | 16 | 16.8 | 17 | 23 | 27.9 | 10.3 | 4.7 | 1.7 | 1.7 | 104 | 42 | 46 | 48 | 73 | 85 | 31 | 14 | 5 | 5 |

| Peak Power Output | | | | | | | | | | 1 min. 5.74 w/kg | | | | | | | | | | 10 min. 5.23 w/kg | | | | | | | | | | Surges: | | | Hydration & Energy Status | | | | | | |
|-------------------|----------|-----------------------|-----|-----|-----|-----|-----|------|-----|--------------------------|----|-----|----|-----|----|------|---|-----------------------------|-----|-------------------|-------------|-------------|----------|--------|-------------|----------|--|--|--|---------|--|--|---------------------------|--|--|--|--|--|--|
| | | Average Power (watts) | | | | | | | | Distance from Start (km) | | | | | | | | # of surges > than w/kg of: | | | Weight (kg) | | | | Bottle | Sweat | | | | | | | | | | | | | |
| | | Sec | | Min | | | | Hour | | Sec | | Min | | | | Hour | | | | | Pre | Post | Δ | % Δ | Count | Loss (l) | | | | | | | | | | | | | |
| | | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | 5 | 30 | 1 | 5 | 10 | 30 | 1 | 2 | > 6 | > 8 | > 10 | 69 | 68 | 1 | 1.4493 | 20 | 10.6 | | | | | | | | | | | | | |
| Fader | Today | 828 | 722 | 478 | 406 | 378 | 358 | 344 | n | 136 | 27 | 21 | 20 | 159 | 20 | 20 | n | | | | Est | Sweat Rates | | | % | | | | | | | | | | | | | | |
| | Tour Avs | | | | | | | | n | | | | | | | | n | | | | Loss (l) | 1/lr | 1/mjoule | GME | Kcals | | | | | | | | | | | | | | |
| | Tour Min | 825 | 528 | 471 | 340 | 296 | 252 | 214 | n | | | | | | | | n | 201 | 49 | 14 | 7.27 | 1.84 | 1.97 | 24 | 5354.156713 | | | | | | | | | | | | | | |
| | Tour Max | 972 | 912 | 674 | 465 | 426 | 377 | 346 | n | | | | | | | | n | 487 | 141 | 43 | | | | | | | | | | | | | | | | | | | |
| Fader | Today | | | | | | | | | | | | | | | | | | | Race Food Eaten: | | | | | | | | | | | | | | | | | | | |
| | Tour Avs | 841 | 555 | 479 | 400 | 360 | 314 | 283 | 255 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Min | 731 | 465 | 403 | 308 | 283 | 249 | 231 | 193 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Tour Max | 965 | 645 | 555 | 478 | 435 | 386 | 359 | 315 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

| Climb/Feature: | Start (km) | Total Elevation Gain | | | | Calc Grade | Stage Elev | Top Elev | Total Gain | Time | | Speed km/hr | VAM m/hr | Estimated Power | | | | Actual | | % Diff |
|---------------------------|------------|----------------------|-------|-------|-----------|------------|------------|----------|------------|------|-----|-------------|----------|-----------------|------|------|-------|--------|------|-------------|
| | | Start | Top | Total | % | | | | | 44hr | 3hr | | | Roll | Aero | Grav | Total | Power | %kg | |
| | | 999 | 999 | 999 | 999 | | | | | 34hr | 3hr | | | | | | | | | |
| 1. Col du Galibier | 2.7 | 45.5 | 42.8 | 4.5 | 4.5045632 | | 720 | 2646 | 1926 | 96 | 32 | 26.60221 | 1197 | 28 | 75 | 252 | 351 | 333 | 4.79 | 6.12343085 |
| 2. Col de La Croix de Fer | 103.8 | 126.5 | 22.7 | 6.9 | 6.8974059 | | 505 | 2067 | 1562 | 47 | 35 | 20.16284 | 1387 | 21 | 33 | 292 | 346 | 334 | 4.81 | 3.432036413 |
| 3. Col du Mollard | 140.7 | 146.5 | 5.8 | 6.8 | 6.8036233 | | 1244.3 | 1638 | 393.7 | 16 | 29 | 21.11223 | 1433 | 22 | 37 | 302 | 361 | 348 | 5.01 | 3.680263055 |
| 4. La Toussuire | 163 | 182 | 18.7 | 5.9 | 5.9033076 | | 603 | 1705 | 1102 | 51 | 202 | 20.63765 | 1216 | 22 | 35 | 256 | 313 | 310 | 4.46 | 0.852670958 |
| Toussuire first 3.12 km | 163.3 | 166.416 | 3.116 | | 7.7898956 | | 603 | 845 | 242 | 8 | 39 | 20.81187 | 1616 | 22 | 36 | 340 | 398 | 382 | 5.50 | 4.018823171 |
| Toussuire to 6.24 km | 166.4 | 169.532 | 3.116 | | 7.7251305 | | 845 | 1085 | 240 | 9 | 51 | 18.98071 | 1462 | 20 | 27 | 308 | 357 | 342 | 4.92 | 3.642213029 |
| Toussuire to 9.36 km | 169.5 | 172.648 | 3.116 | | 7.7251305 | | 1085 | 1325 | 240 | 12 | 26 | 15.037 | 1158 | 16 | 13 | 244 | 273 | 269 | 3.87 | 1.531307335 |
| Toussuire to 12.48 km | 172.6 | 175.764 | 3.116 | | 7.0779971 | | 1325 | 1545 | 220 | 10 | 36 | 17.63774 | 1245 | 19 | 22 | 262 | 303 | 302 | 4.35 | 0.17772058 |
| Toussuire to 15.60 km | 175.8 | 178.88 | 3.116 | | 2.7288717 | | 1545 | 1630 | 85 | 6 | 30 | 28.76308 | 784.6 | 30 | 94 | 165 | 290 | | 0.00 | 100 |
| Toussuire to 18.72 km | 178.9 | 181.996 | 3.116 | | 2.4076295 | | 1630 | 1705 | 75 | 6 | 0 | 31.16 | 750 | 33 | 120 | 158 | 313 | | 0.00 | 100 |





Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstenediols

Cedric H.L. Shackleton,* Andy Phillips,† Tony Chang,* and Ye Li‡

*Children's Hospital Oakland Research Institute, Oakland, California, USA; †Micromass UK Limited, Manchester, England, UK; and, ‡National Research Institute of Sports Medicine, Beijing, China

A gas chromatographic combustion isotope ratio mass spectrometric (GC/C/IRMS) method was used for studying the incorporation of exogenous testosterone enanthate into excreted urinary 5 α - and 5 β -androstane-3 α ,17 β -diols. A multistep but straightforward work-up procedure produced a simple GC chromatogram of urinary steroid acetates composed principally of two androstenediols and pregnanediol. It is anticipated that such a method may form the basis of a doping control test for testosterone that could be used as a primary method during major sporting events or alternatively as a verification technique. Urine samples from five individuals were collected before and after administration of testosterone enanthate (250 mg). The $\delta^{13}\text{C}$ ‰ value of androstenediols was around -26 to -28 during the baseline period and decreased to about -29 to -30 in the days following synthetic testosterone administration. One of the other major steroids in the chromatogram, pregnanediol, was utilized as the "internal standard," because its $\delta^{13}\text{C}$ ‰ values did not markedly change following testosterone administration, remaining at -25 to -27. In all subjects studied, the $\delta^{13}\text{C}$ ‰ values for androstenediols were reduced sufficiently over 8 days to confirm administration of synthetic testosterone. Although steroids isolated from urine of normal individuals from 12 different countries gave values between -24 and -28, this seemed not to be related to nationality or region. The most likely variable is the proportion of plants with low and high carbon 13 content in the diet. This variable is likely to be more affected by individual food preferences than broad ethnic food divisions. In this paper, we propose a ratio of $\delta^{13}\text{C}$ ‰ for androstenediols to pregnanediol as a useful discriminant of testosterone misuse, a value above 1.1:1.0 being indicative of such misuse. The work-up procedure was designed for batch analysis and to use only simple techniques, rather than employ further instrumentation, such as high-performance liquid chromatography (HPLC), in purifying steroids for GC/C/IRMS. (Steroids 62:379-387, 1997) © 1997 by Elsevier Science Inc.

Keywords: isotope ratio mass spectrometry (IRMS); doping control; testosterone

Introduction

The increasing need in sport for proving testosterone misuse requires new methodologies. For many years, a testosterone/epitestosterone (T/E) excretion ratio determined by gas chromatography-mass spectroscopy (GC-MS) of greater than 6:1 has been used as the hallmark for confirmation of drug administration,¹ but this method is fallible. For one thing, occasional drug-free individuals give a ratio >6, and

a high ratio can also be adjusted downward by simultaneous administration of epitestosterone. We have found that in eight Chinese subjects given 250 mg testosterone enanthate, only three gave T/E values >6 on more than 1 day, demonstrating a high rate of false negatives, at least in this racial group.

In 1990, Southan and co-workers² used isotope ratio mass spectrometry (IRMS) to show that synthetic testosterone had a different ^{13}C content than endogenous hormone. This is a reflection of the origin of the materials, because all testosterone, both endogenous and synthetic, is ultimately of plant origin. Gonadal testosterone is made from precursor molecules derived from a wide variety of vegetable mate-

Address reprint requests to Cedric H.L. Shackleton, Children's Hospital Oakland Research Institute, 747 52nd Street, Oakland, CA 94609, USA. Received July 25, 1996; accepted November 4, 1996.

Steroids 62:379-387, 1997

© 1997 by Elsevier Science Inc.

655 Avenue of the Americas, New York, NY 10010

GDC01098

0039-128X/97/\$17.00
PII S0039-128X(96)00253-X

rials eaten by humans or by the animals humans eat. Individual plants discriminate to different degrees against ^{13}C , and particular species are known to have high or low levels of ^{13}C in their biomolecules.³ In human bodies, the ^{13}C content, therefore, reflects an average of the ^{13}C content of all the plant material eaten by humans and our animal protein providers. Synthetic testosterone, in contrast, is generally made from a single plant species, mostly soy, so the ^{13}C content has a defined value reflecting the relatively low ^{13}C content of this plant. Thus, a significant difference in ^{13}C content between gonadal and soy testosterone could provide the basis for developing a definitive test for hormone misuse. Differences in carbon isotope ratios referred to by the symbol δ , defined as the difference in isotope ratio between the sample and an international carbonate standard "PDB." Although this is the accepted standard, for common usage a calibrated international standard of CO_2 is used. The values reported for δ carbon isotope ratios are

$$\delta^{13}\text{C}/\text{‰} = \left(\frac{\text{Ratio sample} - \text{Ratio PDB}}{\text{Ratio PDB}} \right) \times 1000$$

Becchi and coinvestigators have published pioneering studies on development of a method employing IRMS for determination of carbon isotope ratio of testosterone extracted from urine.^{4,5} They demonstrated that, providing sufficient urine was available, the endogenous or exogenous origin of testosterone could be readily determined. A major remaining problem demonstrated by these studies relates to sensitivity of the analysis, because the quantity of urine collected from athletes is relatively small (about 75 mL), and this is divided into two, a primary (A) and a secondary sample (B). On each sample, nonsteroidal drug metabolites must be analyzed, as well as anabolic steroid screening and measurement of the T/E ratio.

We have attempted to improve the methodology to allow more sensitive analysis. It was decided to forgo any attempt to analyze testosterone itself and to concentrate on analysis of its metabolites 5 α -androstane-3 α ,17 β -diol (5 α AD) and 5 β -androstane-3 α ,17 β -diol (5 β AD). Our overall objective was to easily produce a single sample for analysis containing a few defined steroids to include the androstanediols and steroids we call "endogenous reference compounds" (ERCs). An ERC, in this instance, is a steroid whose carbon isotope ratio could not be altered through administration of exogenous testosterone. Aguilera, Becchi, and co-workers in their most recent publication use cholesterol and 5-androstene-3 β ,17 β -diol as ERC.⁵ To achieve our overall objective, we developed simple methodology adaptable to batch analysis, which required no liquid chromatographic (HPLC) instrumentation. Using this methodology, we determined the $\delta^{13}\text{C}/\text{‰}$ of the androstanediols present in urine following administration of testosterone enanthate to five volunteers. This communication presents the results of this study.

Experimental

Materials

Testosterone enanthate, Testoviron depot® was obtained from Schering, Japan. Reference steroids were obtained from Sigma (St.

Louis, Missouri, USA), which was also the supplier of Girard reagent T (carboxymethyl, trimethyl ammonium chloride hydrazide) and sodium bismuthate. Sephadex LH 20 was a product of Pharmacia AB and Sep-pak® cartridges, a product of Waters Corp. (Milford, Massachusetts, USA). β -glucuronidase/aryl sulfatase was obtained from Sigma (Type H1) and Boehringer Mannheim (Mannheim, Germany). Solvents were of analytical grade.

Individuals studied

Eight Chinese male subjects aged 19–22 were studied, although GC/C/IRMS analysis was only conducted on five. Permission for undertaking these experiments was obtained from the Chinese National Research Institute of Sports Medicine, and consent was obtained from the participants. Spot morning urine samples were collected for 2 days prior to an intramuscular injection of 250 mg testosterone enanthate. Two urine samples (0–8 h and 8–24 h) were collected for the first 4 days after administration, although only aliquots of the early morning sample were subject to analysis. Morning spot urine samples were collected on the 5–9th days after administration and on days 11, 13, and 15.

Determination of testosterone/epitestosterone ratio (T/E)

Urinary testosterone and epitestosterone were quantified in all the samples using an adaptation of the method of Donike et al.⁶ These measurements were carried out by one of us (YL) in China at the National Institute of Sports Medicine. T/E ratios were then determined.

Preparation of steroid extract for GC/C/IRMS

A flowsheet summarizing the methodology is shown in Figure 1. Urine (typically 25 mL) was extracted by Sep-pak® cartridge according to the method of Shackleton and Whitney.⁷ Once dried, the extract was dissolved in 3 mL 0.1 M acetate buffer pH 5 and *Helix pomatia*-derived β -glucuronidase/sulfatase (12 mg Sigma

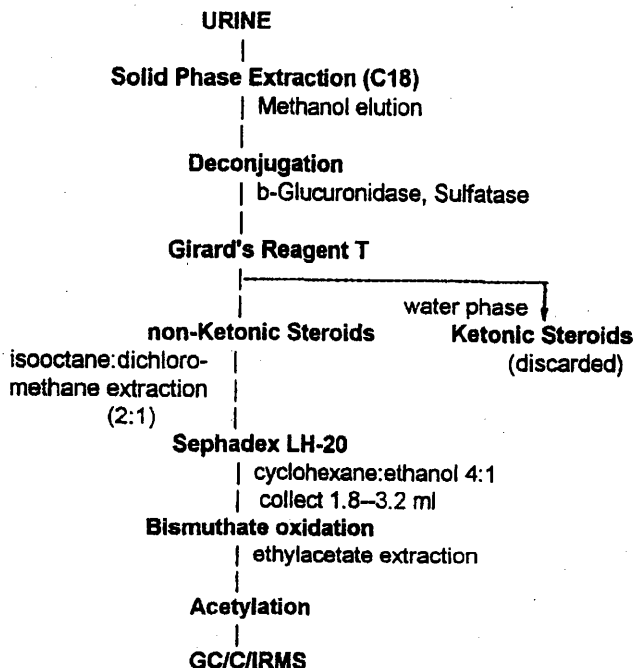


Figure 1 Flow sheet of the urinary extraction method.

type H1 powder, 100 μL Boehringer liquid enzyme) was added. Hydrolysis was allowed to proceed for 3 h at 55°C. A Girard separation was carried out to separate carbonyl-containing steroids (ketonic) from noncarbonyl-containing (nonketonic) steroids.^{8,9} To the hydrolyzed urine mixture, 2 mL glacial acetic acid and 100 mg Girard reagent T was added. The solutions were placed in an oven at 100°C for 30 min. The nonketonic steroids were extracted by 2 \times 5 mL isooctane: dichloromethane 2:1 (v/v), and the solvent was dried. Small columns of 0.5 Sephadex LH-20 were prepared in Pasteur pipettes, the Sephadex being allowed to swell in the cyclohexane: ethanol (4:1) solvent system before preparation.¹⁰ The steroid extract dissolved in 100 μL of the same solvent mixture was added to these columns. Solvent eluting between 1.8 and 3.2 mL was collected and dried. Acetic acid (0.1 mL), water (0.1 mL), and 5 mg sodium bismuthate were added.⁹ Oxidation was allowed to proceed for 2 h, and after neutralization (0.5 mL of 0.5 M acetate buffer), the mixture was extracted with 4 mL ethyl acetate. After drying, steroid acetates were prepared overnight with 50 μL acetic anhydride and 50 μL pyridine. The acetates were analyzed by GC/C/IRMS.

Gas chromatography combustion isotope mass spectrometry (GC/C/IRMS)

A schematic representation of the GC/C/IRMS instrumentation is illustrated in Figure 2. The acetylated steroid samples were kept refrigerated until analysis. Cyclohexane (20 μL ; 99.9% pure from Sigma Chemicals 27-0625-8) was added to each vial, and one-tenth (2 μL) of each sample was injected splitless onto a J&W 30 m DB17 capillary column housed in a Fisons 8000 series GC. The injector was kept at 220°C. The temperature program was as follows: starting temperature 50°C (1 min), followed by rapid temperature increase (25° min) to 300°C, where it was held for 15 min.

The separated components were heart-split into the combustion furnace filled with copper oxide wires (Elemental Microanalysis Limited, UK) held at 850°C. The combustion gases were passed through a nafion membrane water removal trap, and the remaining CO_2 was analyzed on a Micromass isochrom isotope mass spectrometer. The mass spectrometer consisted of an electron impact source running at 400 μA current, the ionized CO_2 gas, was focused by a magnet onto three Faraday collectors. The ions collected were those at masses 44, 45, and 46. The Micromass data system calculated the areas of the beams and subtracted any background; whereupon, calculation of the ^{13}C δ values for the successive peaks were carried out.

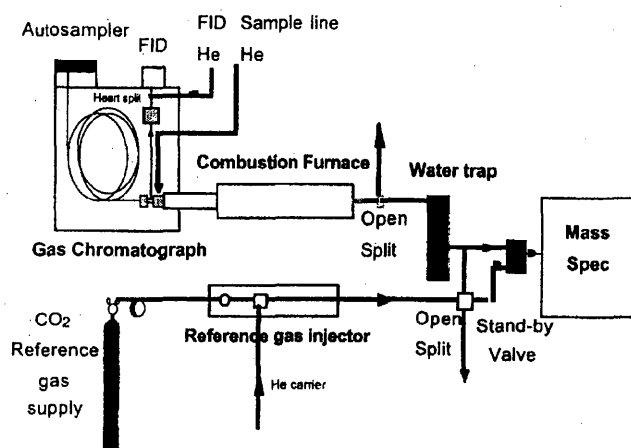


Figure 2 Schematic of the Micromass GC/C/IRMS instrument.

Results and discussion

Evaluation of gas chromatographic columns

The objective of the study was to measure $\delta^{13}\text{C}/100$ for urinary androstane diols formed as metabolites of testosterone. In our initial studies (Subject 1), we undertook chromatography on DB1 capillary columns but did not get resolution of the two diacetylated epimers (Figure 3A). Later employment of a DB17 column (Figure 3B) allowed separation of the epimers as well as the ERC, pregnane diol diacetate, and pregnane triol diacetate (Figure 3B).

Confirmation of identity of steroids in extracts

Prior to sending the first samples for GC/C/IRMS analyses in England, the identities of the principal components of the chromatogram were confirmed by GC/MS. This was carried out on a Hewlett-Packard 5970 instrument housing a 15 meter DB1 capillary column. The peaks chosen for GC/C/IRMS analyses had retention times and electron impact mass spectra identical to those of 5 α - and 5 β -androstane diol diacetate and pregnane diol diacetate. Reference steroids for these compounds were also analyzed on the GC/C/IRMS instrument using both DB1 and DB17 columns, and these gave identical retention times to the urinary steroids. Pregnane triol could also be analyzed by GC/C/IRMS.

Achievement of work-up procedure objective

The method developed and utilized had the following qualities. First, the Girard separation almost completely removed carbonyl containing steroids from the hydrolyzed extracts, which probably represent 75% of urinary steroids. Exceptions may be the 11-carbonyl containing steroids that probably do not react because of the hindered nature of that functional group. Second, a crude micro Sephadex LH-20 column separation effectively produced a fraction containing steroids with two and three functional groups. Third, sodium bismuthate oxidation was designed into the procedure as a means of removing remaining long-retention time pregnane metabolites, thus allowing shorter periods between injection. Many of the quantitatively more important metabolites are converted into 17-oxygenated C_{19} steroids by the procedure. Fourth, acetylation provided steroids with good gas chromatographic properties that were readily separated. Fifth, despite the complexity of the steroid fraction of urine, the final chromatogram was simple and composed of only a few peaks for which $\delta^{13}\text{C}/100$ could be determined with accuracy.

$\delta^{13}\text{C}/100$ value of the synthetic testosterone

Testosterone acetate prepared from the Japanese testosterone enanthate used for injection in these studies gave a $^{13}\text{C}/100$ value of -30.41 , a value close to the lowest value obtained for androstane diol diacetate measurements obtained in the subjects studied following testosterone administration.

We also analyzed five other current products and one synthetic sample made more than 40 years ago. The following results were obtained: testosterone of Chinese manufacture -30.40 ; U.S. manufacture -30.38 ; two Czech products,

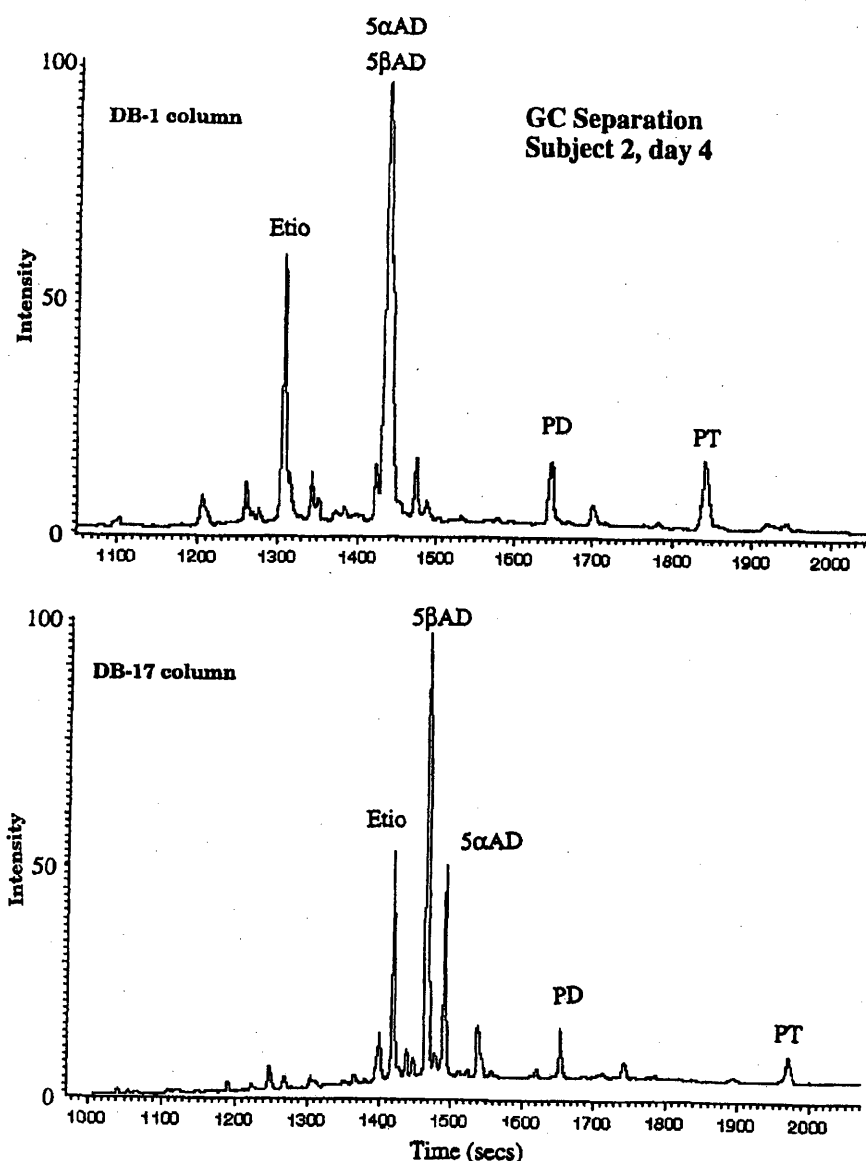


Figure 3 Gas chromatographic separation (on GC/C/IRMS instrument) of acetylated urinary steroid fraction. This sample was initially separated on a DB1 column, which failed to resolve the 5 α - and 5 β -androstanediols (upper panel). Later employment of a DB17 column permitted individual measurement of the epimers (lower panel). Both columns also resolved etiocholanolone, produced by bismuthate oxidation of pregnanetriol, pregnanediol, and nonoxidized pregnanetriol.

–29.28 and –29.15, respectively; and one Russian –30.22. The U.S. sample of more than 40 years old gave a value of –33.18, probably reflecting the different plant origin of synthetic steroids made long ago. We believe that testosterone is currently made mostly from soy by-products, although originally sterol constituents of the Mexican yam were the primary precursors.

Results for individual subjects

With the exception of Subject 1, a DB17 capillary column was utilized that allowed separate analysis of 5 α - and 5 β -androstanediols. Graphs illustrating the $\delta^{13}\text{C}/100$ results are shown in Figure 4A–F. In all cases, the $\delta^{13}\text{C}/100$ values for

the androstanediols fell significantly following testosterone administration.

Figure 4 illustrates two other features. One is the adequacy of making single measurements of $\delta^{13}\text{C}/100$ for each sample. Panel B and C show results obtained by plotting the average of duplicate measurements (B) and of the *first* measurement of the duplicate pair (C). Essentially these graphs are identical, suggesting duplicate GC/C/IRMS analyses of the same preparative extract are unnecessary. The second feature relates to the trend of slightly increasing δ values for pregnanediol during the study period of Subject 3 (Figure 4D). Because this is not caused by random irreproducibility we proposed that the individual changed his diet substantially to foodstuffs with higher $\delta^{13}\text{C}/100$ values.

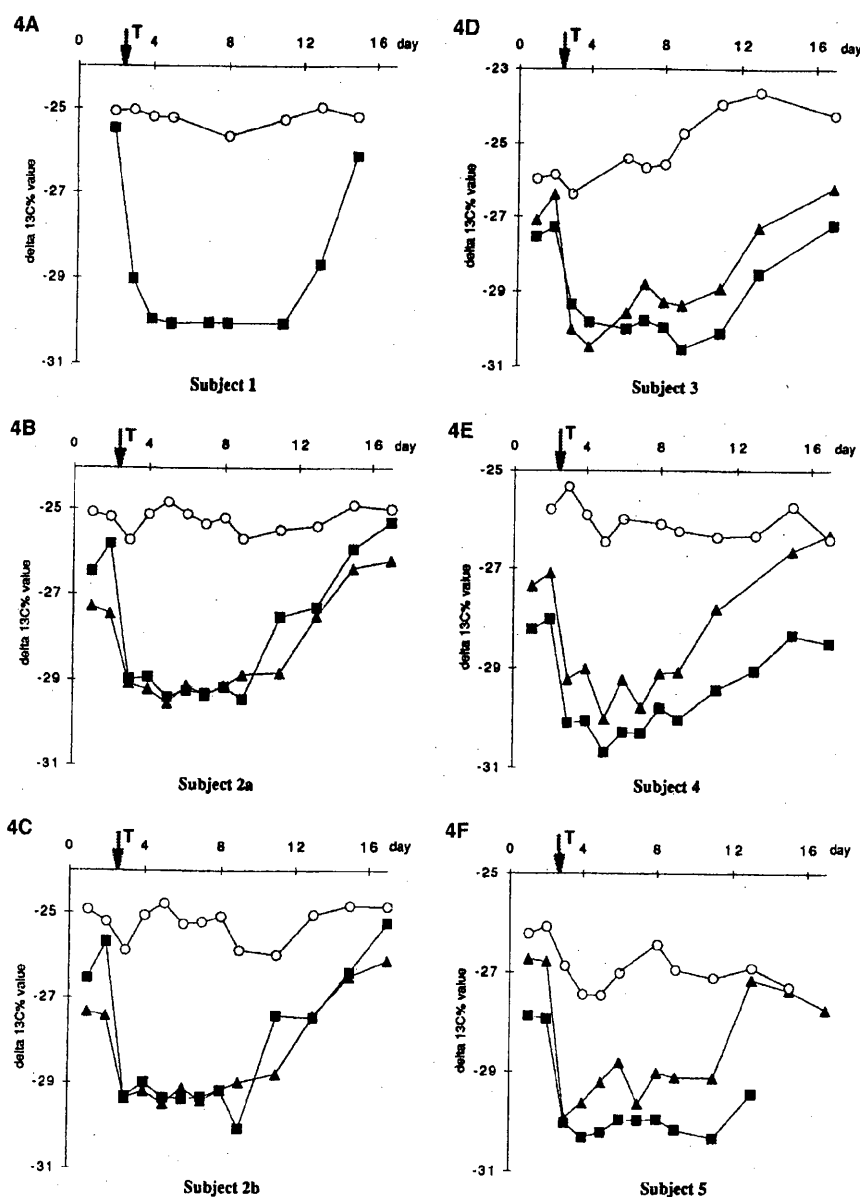


Figure 4 Values of $\delta^{13}\text{C}^0/00$ for all five subjects throughout the study period. The open circles represent pregnanediol, the closed rectangles 5 β AD and closed triangles 5 α AD. 4A shows the values obtained for Subject 1 in which only one baseline sample was analyzed, the testosterone being administered on the 2nd day of study. Both androstenediols were measured together on a DB1 column, 4B shows plots for Subject 2 obtained by averaging values for the duplicate runs, and 4C shows equivalent plots through only using the first analysis. 4C, 4D, and 4E are plots of Subjects 3, 4, and 5. For Subjects 2–5 the testosterone was administered after the second baseline sample (day 3 of study).

Designing a method for universal use demands final agreement of numerative values above and below which individuals are considered to have or to have not illicitly used a drug. In our studies with the Chinese subjects, it can be stated that for the five individuals, none had androstenediol $\delta^{13}\text{C}^0/00$ values less than -28.3 during the control period, and establishing a conservative cut-off value of -29.0 would clearly pick up all drug users for about 7 days after administration. Additionally, we considered that measurement of analyte/ECR ratios could allow numerative values to be established that are to some degree independent of variables introduced through using the methodology in different laboratories with different instrumen-

tation. In Figure 5A, we show the $\delta^{13}\text{C}^0/00$ ratio of combined androstenediols (average of 5 α - and 5 β -) to pregnanediol (PD) for the five individuals studied so far. All baseline ratios fell below 1.08, and from these data, we would suggest that a ratio of greater than 1.1 could be used to confirm testosterone administration. When we averaged the data for the five individuals, we found that the AD/PD ratio value of 1.1 was exceeded for 11 days (Figure 5B). Because a combined androstenediol measurement is carried out in the Aguilera et al. studies,⁵ comparable data could be prepared from their measurements, although in their case, cholesterol would be the ERC used for ratio determination.

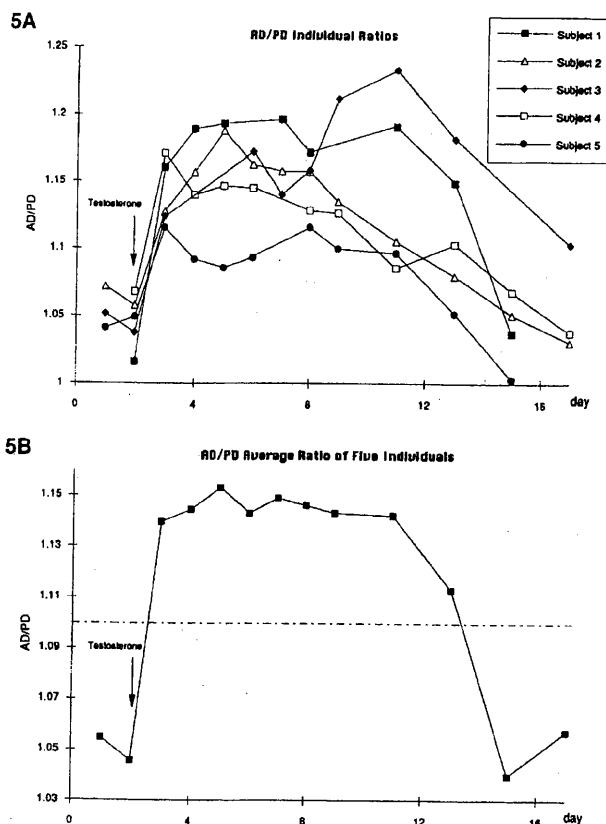


Figure 5 Androstanediol/pregnanediol ratio. A) ratios of $\delta^{13}\text{C}/00$ for the androstanediols (mean of 5α and β) to pregnanediol for the five subjects; B) average of the ratio for the five subjects for each day; the dotted line represents the proposed discriminant value (1.10) for confirmation of testosterone administration.

Reproducibility of isotope measurements and accuracy

For the five subjects studied, ideally 26 or more isotope measurements were made for each steroid; i.e., 13 duplicate analyses. A few samples were lost or contaminated, and occasionally only single measurements were made on a sample. As a representative analysis, Table 1 gives all of the measurements for Subject 2. Using these data, there are two features of accuracy and reproducibility that can be quantified. One is the variation in δ measurement between duplicate analyses of the same sample and the other the overall variation occurring during analyses of a complete series from one individual. For duplicates, the reproducibility was excellent and did not differ significantly between the five subjects studied. Considering the data shown in Table 1, averages and standard deviations were measured for each duplicate for the two androstanediol and pregnanediol diastereates that give values in the $\delta^{13}\text{C}/00$ -23 to -30 range. Means were then determined of the standard deviations. For pregnanediol, the mean standard deviation between duplicates was ± 0.21 with a range of ± 0.02 to ± 0.71 . Comparable results were obtained for the androstanediols (± 0.11 for 5α -androstanediol and ± 0.25 for 5β -androstanediol). These results were considered to represent excellent reproducibility and (as previously noted) suggested that the meth-

odology could be used with *single* determinations by GC/C/IRMS of $\delta^{13}\text{C}/00$ for each steroid. This was illustrated graphically in Figure 4B and C, which showed both the $\delta^{13}\text{C}/00$ values for the average of duplicates and for the first analysis. The graphs were essentially identical.

Within a 17-day series of datapoints for duplicate assays for a single individual, the results show considerably less reproducibility—notice the peaks and valleys of the pregnanediol plots in Figure 4. In any physicochemical analyses, it is, of course, possible to have excellent reproducibility but compromised values. For accuracy and reproducibility assessment between a series of different samples, we use the δ measurement of the ERC pregnanediol. The administration of testosterone should in no way change the $\delta^{13}\text{C}/00$ value for pregnanediol that has to be produced from cholesterol via several intermediates. In a perfect analysis, the measured ^{13}C content of pregnanediol would not be expected to change during the 17-day study period unless, of course, the individual alters his or her diet markedly during the study period. Even with dietary modification, the ^{13}C content of body molecular constituents would be expected to change only slowly.

The highest and lowest duplicate average $\delta^{13}\text{C}/00$ values for pregnanediol for the five subjects differed by -0.92 to -2.96 . As previously mentioned, with the exception of Subject 2, the average duplicate values seemed to rise and fall randomly during the 17-day study period. What

Table 1 $\delta^{13}\text{C}/00$ values obtained from duplicate GC/C/IRMS analyses for Subject 2 before and following testosterone administration

| | Subject 2 | | |
|--------------------|-------------------|--------------------|--------|
| | $5\beta\text{AD}$ | $5\alpha\text{AD}$ | PD |
| Day 1 | -26.55 | -27.33 | -24.93 |
| | -26.15 | -27.20 | -25.34 |
| | -26.66 | -27.28 | -25.15 |
| Day 2 | -25.68 | -27.42 | -25.20 |
| | -25.95 | -27.49 | -25.17 |
| Day 3 ^a | -29.33 | -29.36 | -25.88 |
| | -28.63 | -28.81 | -25.61 |
| Day 4 | -28.99 | -29.19 | -25.06 |
| | -28.88 | -29.24 | -25.21 |
| Day 5 | -29.34 | -29.49 | -24.78 |
| | -29.48 | -29.61 | -24.87 |
| Day 6 | -29.37 | -29.11 | -25.26 |
| | -29.12 | -29.16 | -24.97 |
| Day 7 | -29.33 | -29.42 | -25.22 |
| | -29.31 | -29.28 | -25.46 |
| Day 8 | -29.16 | -29.16 | -25.09 |
| | -29.18 | -29.14 | -25.30 |
| Day 9 | -30.06 | -28.98 | -25.88 |
| | -28.83 | -28.78 | -25.52 |
| Day 11 | -27.41 | -28.78 | -25.99 |
| | -27.63 | -28.88 | -24.99 |
| Day 13 | -27.47 | -27.42 | -25.05 |
| | -27.12 | -27.59 | -25.72 |
| Day 15 | -26.39 | -26.50 | -24.84 |
| | -25.50 | -26.28 | -24.96 |
| Day 17 | -25.24 | -26.12 | -24.86 |
| | -25.36 | -26.32 | -25.13 |

Abbreviations: $5\beta\text{AD}$, 5β -androstane- $3\alpha,17\beta$ -diol; $5\alpha\text{AD}$, 5α -androstane- $3\alpha,17\beta$ -diol and PD, pregnanediol

^aThe first collection following testosterone administration.

is the source of this irreproducibility? Because we know that the reproducibility of $\delta^{13}\text{C}/00$ measurements of individual steroids in duplicate GC/C/IRMS runs is excellent, we must assume that the measured $\delta^{13}\text{C}/00$ values for individual chromatographic peaks are accurate. If these are accurate, then any difference in the value from the "true" value for the steroid determined must represent some minor contamination of the steroid peak by components with greater or less $\delta^{13}\text{C}/00$ values. We have proposed that the trend to increasing values for Subject 2 may be because of dietary changes toward foodstuffs with greater ^{13}C content.

These minor irreproducibilities, which typically give rise to a range of about -1.5 in the δ value for all datapoints during the study period, will not affect the outcome of a drug test, because in no cases do the lowest values breach the discriminant value for the positives.

Testosterone/epitestosterone ratios

Table 2 gives the T/E values for five individuals studied here (Subjects 1–5) and three other subjects whose steroids were not analyzed by the IRMS technique (Subjects 6–8). The data show that the maximal increase in the ratio for each of the individuals was between 12 and 68 times baseline level, although it was noticeable that only 4 of the 8 subjects gave T/E values greater than 6 and 1 of these only modestly exceeded it on one occasion (T/E 6.69). The T/E ratio of the five subjects studied here have been included in Figure 5C as compared to the $\delta^{13}\text{C}/00$ AD/PD ratio for the same individuals.

$\delta^{13}\text{C}/00$ steroid values for different nationalities

Figure 6 shows the $\delta^{13}\text{C}/00$ values for duplicate analyses of the androstane diols and PD from 15 individuals of 11 different nationalities in addition to the Chinese. In some cases, it was possible only to obtain values for the 5β androstane diols because of the low amounts of 5α AD present. The results demonstrate that there are differences among racially and regionally varied individuals with undetermined diet, but we must remember that part of this variation may be attributable to the analytical inaccuracies previously discussed. However, all values were greater than -28.2 , which should be compared to the values of less than -29.2

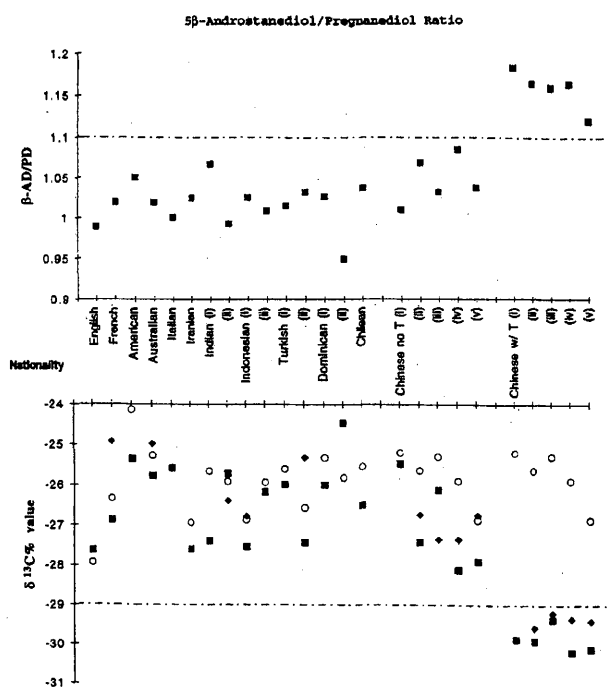


Figure 6 The lower panel shows the $\delta^{13}\text{C}/00$ values for the androstane diols and pregnane diols for individuals of different nationality, while the upper panel shows the $5\beta\text{AD}/\text{PD}$ ratio. The open circles represent PD, the closed diamonds $5\alpha\text{AD}$ and closed rectangles $5\beta\text{AD}$. The baseline values for the Chinese represent the mean of the two measurements, while the Chinese "testosterone" values represent the average of the 7 days after drug administration. Pregnanediol was below detection level in the Chilean urine, so pregnanetriol values were reported instead.

found in the subjects studied following testosterone administration. The mean value for the $5\beta\text{AD}/\text{PD}$ ratio was 1.02 ± 0.03 with a range of 0.95 to 1.07, which compares to the average value for the Chinese baseline samples of 1.05 ± 0.03 (range 1.01 to 1.08). The ratio values for the testosterone administered subjects were all >1.15 in the first days after injection. These data support the previous cut-off ratio value of 1.1:1.0 suggested on the basis of studying the Chinese subjects before and after testosterone administration.

There are notable features with regard to the ^{13}C content

Table 2 Urinary testosterone/epitestosterone ratios for the eight subjects

| Study day | Subject 1 | Subject 2 | Subject 3 | Subject 4 | Subject 5 | Subject 6 | Subject 7 | Subject 8 |
|----------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 1 | 0.26 | 1.28 | 4.69 | 0.10 | 0.12 | 0.17 | 0.08 | 0.11 |
| 2 | 0.33 | 1.48 | 5.26 | 0.11 | 0.11 | 0.22 | 0.06 | 0.11 |
| 3 ^a | 0.65 | 8.77 | 29.3 | 0.26 | 0.7 | 1 | 1.07 | 0.42 |
| 4 | 1.95 | 24.2 | 36.9 | 1.77 | 6.69 | 4.13 | 1.36 | 2.98 |
| 5 | 2.81 | 45.8 | 41.8 | 2.01 | 5.91 | 3.53 | 1.53 | 7.23 |
| 6 | 2.79 | 33.2 | 61.8 | 1.96 | 3.71 | 3.10 | 1.45 | 7.48 |
| 7 | 3.03 | 15.4 | 54.9 | 1.91 | 4.24 | 3.65 | 1.68 | 7.16 |
| 8 | 4.13 | 36.4 | 57.7 | 1.48 | 5.95 | 3.08 | 0.97 | 4.89 |
| 9 | 5.3 | 20.3 | 39.7 | 1.51 | 5.78 | 1.43 | 1.04 | 3.81 |
| 11 | 3.2 | 26.5 | 33.0 | 0.58 | 3.69 | 0.97 | 0.40 | 3.69 |
| 13 | 0.77 | 2.63 | 3.42 | 0.68 | 3.41 | 0.20 | 0.07 | 1.93 |
| 15 | 0.22 | 0.99 | 4.37 | 0.19 | 2.27 | 0.15 | 0.06 | 0.21 |
| 17 | 0.26 | 2.02 | 4.80 | 0.13 | 0.21 | 0.20 | 0.08 | 0.08 |

^aDay of testosterone administration.

of steroids in urine from individuals of different countries. One is that there is a considerable spread in values but one that is unrelated to race and "typical regional diets," at least based on the small sample studied. The lowest and highest $\delta^{13}\text{C}^0/00$ among this group differed by 4 units (-24 to -28); however, both these sets of values came from Caucasians. The remaining samples from different nationalities and ethnic groups fell within this range, a particularly important finding with respect to the current study, because it suggests that the results obtained following testosterone administration are universally applicable.

Diet of individuals must have the greatest part to play in the establishment of an individual's $\delta^{13}\text{C}$ value. Because all food originates as plant life (including animal protein), it was interesting to research what is known of $\delta^{13}\text{C}^0/00$ values for dietary plants. Smith and Epstein³ published the ^{13}C content of 104 plants, but relatively few were commonly used foodstuffs. A few were high $^{13}\text{C}/^{12}\text{C}$ plants (corn, sugar cane, grain, and sorghum) averaging around $\delta^{13}\text{C}^0/00$ -14 , but most were categorized as a separate group of low $\delta^{13}\text{C}^0/00$ species having values between -23 and -30 . These include dietary oil precursors (olive, sunflower, and castor) and various vegetables (wheat, grass, bamboo, peas, squash, radish, beets, and citrus fruit). Because typical worldwide diets of human and domestic animals use mostly low $\delta^{13}\text{C}^0/00$ plants, we would anticipate that $\delta^{13}\text{C}$ values for excreted steroids would fall in the -23 to -28 range, as found. The $\delta^{13}\text{C}^0/00$ value of an individual plant species does not vary significantly dependent on geographic location,³ and variation only manifests itself in plants grown in urban areas where CO_2 from fossil fuels increases the ^{12}C content slightly. This clearly does not markedly affect agricultural foodstuffs. For the purposes of this assay technique, it is fortuitous that synthetic testosterone is obviously derived from a very low $\delta^{13}\text{C}^0/00$ plant species, otherwise no distinction would be possible between endogenous and exogenous compound.

Practicality of methodology for use in doping control

We compared our $\delta^{13}\text{C}^0/00$ values for androstanediol with those of Aguilera et al., who used similar methodologies but different instrumentation.⁵ They studied individuals of different nationality and ethnicity than ours. For the purposes of this comparison, we averaged our values for 5α - and 5β -androstanediol, because their values were based on measurement of the combination. For baseline samples, we obtained an averaged $\delta^{13}\text{C}^0/00$ of -26.87 , which agreed excellently with Aguilera's⁵ value of -26.52 . For androstanediols measured during testosterone administration, they obtained an average value of -32.44 ; whereas, our value (the mean of duplicates of the lowest values for each individual) was -30.21 . Although our minimum value is not as low as that of Aguilera, it was still well below the baseline level, so it could be used for proving testosterone abuse. The difference in results between the laboratories could be attributed to their use of a testosterone with lower $\delta^{13}\text{C}^0/00$ (its origin was not reported) or unequal calibration of the different instruments used.

Our methodology is multistep, albeit built around low-

technology procedures. Within the technique, there is only one chromatographic separation, and that was designed to produce a broad fraction rather than the isolation of individual steroids. The key to the method is separate isolation of nonketonic steroids, a procedure that in one extraction step removes 75% of unwanted steroids from urine. A second classical procedure, the oxidative removal of side chains from 17-hydroxypregnane steroids removes many of the remaining complex steroids from the mixture, resulting in a cleaner chromatogram, although in the future, we may simplify the method by discontinuing this procedure if satisfactory results can be obtained. Typically, without using any automation in wet chemistry procedures, a batch of samples requires 1 day of technician time to extract and derivatize. The mass spectrometry was automated, and later 20 minutes were required for each run. The maximum number of samples that could be analyzed per 24 h was, therefore, 72.

The length of time that the 250-mg testosterone enanthate dose (equivalent to 180-mg free testosterone) clearly manifested itself in decreased $\delta^{13}\text{C}^0/00$ value was 8–10 days. This compares well with other parameters studied on this cohort. For example, plasma testosterone was measured, and from being grossly elevated in the first few days after administration, it returned to normal by the 8th day, although the steroid itself may well be largely of exogenous origin on this occasion.¹¹ In addition, we report the T/E ratios that are particularly interesting, because essentially only three out of the eight individuals achieve values >6 on more than 1 day, showing a high rate of false negatives. This may not be a universal finding and may well be related to the race (Asian) of the studied individuals. However, it once again emphasizes the importance of developing alternate methodology for proving testosterone misuse. The elevated T/E ratios (although most are <6) fall significantly by the 11th day of the study in all subjects.

We believe that our method based on androstanediol measurement has the needed sensitivity for use in doping control. Although we analyzed all samples in duplicate and plotted the graphs from their averages, individual analyses were equally informative (Figure 4B, C). We would have no hesitation in confirming testosterone administration based on a single measurement. We have been able to get up to five analyses of a single sample using splitless gas chromatographic injection. This represents an equivalent of 5 mL urine for each analysis, which meets sample availability in doping control. Use of a solid injection device for GC, such as glass minivials in a carousel,¹² could improve this sensitivity greatly, because the total sample could be volatilized and passed into the GC/IRMS instrument. Testing of a urine volume of 2 mL by our procedure and the Micromass IRMS instrument would be realistic. Our method has two obvious advantages over that of Aguilera et al.⁵ One is that the two androstanediols are determined separately, so even if one peak was too small or showed evidence of contamination, the second peak could give the required value. A second advantage is that all analytes are measured in the same chromatogram; whereas, in Aguilera et al.'s study, at least two separate GC/C/IRMS analyses are required to measure the isotope ratio in the androstanediols and their

ERC cholesterol acetate. However, in fairness, they did have a more difficult assay through the inclusion of testosterone itself among the analytes.

Manipulating the result of a drug test to give a "negative" result following testosterone administration would be difficult. Although the AD/PD ratio could be normalized by taking commercial pregnanediol (Sigma, $\delta^{13}\text{C}^0/00$ value -32.12) the low "absolute" value for pregnanediol would give the situation away. Similarly, commercial androstane-diols also have very low $\delta^{13}\text{C}^0/00$ values (Sigma -34.00) so self-administration of these compounds would not increase the δ values of testosterone derived metabolites. Probably the only way to fool such a test would be to alter the diet drastically over a long period of time through exclusively ingesting foodstuffs with $\delta^{13}\text{C}^0/00$ values below -29 . False positives could be obtained by ingestion of nonproscribed steroids such as the now commonly used dehydroepiandrosterone (DHEA), which also partially metabolizes to androstane-diols. However, whether testosterone or DHEA was being administered could easily be determined from marked differences in the total urinary steroid profile.

Acknowledgments

These studies were made possible through the generous support of the U.S. Olympic Committee and its representatives Dr. Wade Exum and Dr. James Betts. Financial support was also provided by Children's Hospital Oakland Research Institute. The authors thank Dr. Victor Rogozkin (St. Petersburg), Dr. Lubos Stárka (Prague), and Drs. Xavier de la Torre and Jordi Segura (Barcelona) for donating synthetic testosterone standards. Thanks are also given to individuals from different countries who provided urine samples for analysis. These studies could not have been carried out without the continual mass spectrometric maintenance and training undertaken by Ms Esther Roitman.

References

1. Catlin DH, Cowan DA, De la Torre R, Donike M, Fraisse D, Oftegro H, Hatton CK, Starcevic B, Becchi M, de la Torre X, Norli H, Geyer H, Walker CJ (1996). Urinary testosterone (T) to epitestosterone (E) ratios by GC/MS. I. Initial comparison of uncorrected T/E in six international laboratories. *J Mass Spectrom* **31**:397-402.
2. Southan G, Mallet A, Jumeau J, Craig S, Poojara N, Mitchell D, Wheeler M, Brooks RV (1990). Programme and Abstracts of The 2nd International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, Spain, p. 306.
3. Smith BN, Epstein S (1971). Two categories of $^{13}\text{C}/^{12}\text{C}$ ratios for higher plants. *Plant Physiol* **47**:380-384.
4. Becchi M, Aguilera R, Farizon Y, Flament IM-M, Casabianca H, James P (1994). Gas chromatography/combustion/isotope-ratio mass spectrometry analysis of urinary steroids to detect misuse of testosterone in sport. *Rapid Commun Mass Spectrom* **8**:301-308.
5. Aguilera R, Becchi M, Casabianca H, Hatton CK, Catlin DH, Starcevic B, Pope JHG (1996). Improved method of detection of testosterone abuse by gas chromatography/combustion/isotope ratio mass spectrometry analysis of urinary steroids. *J. Mass Spectrom* **31**:169-176.
6. Donike M, Bärwald KR, Klosterman K, Schänzer W, Zimmerman J (1983). *Sport. Leistung und Gesundheit*, Cologne, Germany, p. 293.
7. Shackleton CHL, Whitney JO (1980). Use of Sep-pak® cartridges for urinary steroids extraction: Evaluation of the method for use prior to gas chromatographic analysis. *Clin Chim Acta* **107**:231-243.
8. Girard A, Sandulesco G (1936). Sur une nouvelle série de réactif du groupe carbonyle, leur utilisation à l'extraction des substances cétoniques et à la caractérisation microchimique des aldehydes et cétones. *Helv Chim Acta* **19**:1095-1107.
9. Bush IE (1961). *The Chromatography of Steroids*. Pergamon Press Ltd., Oxford, UK, pp. 358-371.
10. Setchell KDR, Shackleton CHL (1973). The group separation of plasma and urinary steroids by column chromatography on Sephadex LH-20. *Clin Chim Acta* **47**:381-388.
11. de la Torre X, Segura J, Farré M, Yang Z, Li Y, Wu M (1995). Steroid profile in different ethnic groups after testosterone ethanate administration. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U (eds), *Recent Advances in Doping Analysis*. Sport und Buch Strauß, Köln, Germany, pp. 59-80.
12. Shackleton CHL, Honour JW (1976). Simultaneous estimation of urinary steroids by semiautomated gas chromatography. Investigation of neonatal infants and children with abnormal steroid synthesis. *Clin Chim Acta* **69**:267-283.



ELSEVIER

Journal of Chromatography A, 842 (1999) 351–371

JOURNAL OF
CHROMATOGRAPHY A

Review

Applied gas chromatography coupled to isotope ratio mass spectrometry

W. Meier-Augenstein*

Department of Anatomy and Physiology, University of Dundee, Small's Wynd, Dundee DD1 4HN, Scotland, UK

Abstract

Compound-specific isotope analysis (CSIA) by isotope ratio mass spectrometry (IRMS) following on-line combustion (C) of compounds separated by gas chromatography (GC) is a relatively young analytical method. Due to its ability to measure isotope distribution at natural abundance level with great accuracy and high precision, GC–C–IRMS has increasingly become the method of choice in authenticity control of foodstuffs and determination of origin in archaeology, geochemistry, and environmental chemistry. In combination with stable isotope labelled compounds, GC–C–IRMS is also used more and more in biochemical and biomedical application as it offers a reliable and risk-free alternative to the use of radioactive tracers. The literature on these topics is reviewed from the advent of commercial GC–C–IRMS systems in 1990 up to the beginning of 1998. Demands on sample preparation and quality of GC separation for GC–C–IRMS are discussed also. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Food analysis; Mass spectrometry; Isotope analysis; Environmental analysis; Geochemistry; Detection, GC

Contents

| | |
|--|-----|
| 1. Introduction | 352 |
| 2. Principles of IRMS and GC–C–IRMS | 353 |
| 2.1. IRMS | 353 |
| 2.2. GC–C–IRMS | 353 |
| 2.2.1. Sample preparation | 355 |
| 2.2.1.1. Derivatization | 356 |
| 2.2.2. Isotopic calibration | 356 |
| 2.2.3. HRcGC | 357 |
| 3. High-precision CSIA of ¹³ C isotopic abundance | 358 |
| 3.1. GC–C–IRMS at natural abundance level | 358 |
| 3.1.1. Authenticity | 359 |
| 3.1.1.1. Flavours and fragrances | 359 |
| 3.1.1.2. Wine, fruit juice and honey | 360 |
| 3.1.1.3. Vegetable oils | 361 |

*Fax: +44 1382 345514.

E-mail address: w.meieraugenstein@dundee.ac.uk (W. Meier-Augenstein)

| | |
|---|-----|
| 3.1.1.4. Drugs | 362 |
| 3.1.2. Origin | 362 |
| 3.1.2.1. Geochemistry | 362 |
| 3.1.2.2. Archaeology | 363 |
| 3.1.2.3. Environmental chemistry | 363 |
| 3.2. Tracer studies | 364 |
| 4. Compound-specific isotope analysis of ^{15}N isotopic abundance | 365 |
| 5. Hyphenated techniques | 366 |
| 6. Conclusions | 368 |
| Acknowledgements | 368 |
| References | 368 |

1. Introduction

During the 14 years that followed the first publications reporting the coupling of gas chromatography (GC) to on-line combustion of GC separated compounds to yield CO_2 and N_2 for isotope ratio analysis by a single collector mass spectrometer [1,2], hardly any work was published that made use of this new technique. Drawing on this work, in 1984, Barrie et al. [3] coupled a dual collector mass spectrometer to a GC via an on-line combustion interface, thus permitting continuous recording of $(m+1)/m$ isotope ratios by detecting two successive masses at the same time. Their instrument was the first genuine GC-combustion interfaced-isotope ratio mass spectrometry (GC-C-IRMS) system and produced isotope ratios that were an order of magnitude more precise than obtained from an optimised single collector instrument.

However, it was not until 1990 that GC-C-IRMS instruments became commercially available. Since then, GC-C-IRMS instrumentation has experienced several advances and its application has increased to such an extent that it "could almost be considered a conventional technique" [4].

Tom Brenna of Cornell University's Division of Nutritional Sciences, once said "IRMS is probably the first form of analytical mass spectrometry" (see Ref. [166]). IRMS is certainly a very sensitive detector, able to yield highly precise measurements of isotope ratios with a standard deviation in the range of four to six significant figures. When coupled to a GC system, it enables the analyst to conduct

highly precise compound-specific isotope analysis (CSIA), especially at natural isotopic abundance level. High-precision CSIA at natural abundance level can provide information on biogenetic relation and origin of a given organic compound. Compared with authentic reference data, subtle differences in the isotopic abundance of ^2H , ^{13}C , ^{15}N , or ^{18}O can thus help uncover adulteration of foodstuff or drug abuse in sports to name but a few.

There is also an increasing interest in the application of high-precision CSIA in tracer studies. One area of application is concerned with quantitative studies of biochemical processes such as assimilation/incorporation of nutrients, turnover rates of biologically important molecules, and quantitation of protein synthesis. The other area aims to improve detection limits of bio-organic molecules by using labelled precursor compounds at high enrichment levels.

In either case, high-resolution capillary gas chromatography (HRcGC) is a prerequisite for high-precision CSIA by on-line IRMS. Peak overlap and peak distortion have a detrimental effect on both accuracy and precision of isotope ratio measurements. It is therefore not surprising that scientists working on high-precision CSIA by on-line IRMS invariably employ HRcGC methods.

It is the aim of this article to provide a review over the current spectrum of applied GC-C-IRMS in conjunction with the HRcGC aspects involved. To illustrate as to why the two are inextricably linked, the first section of this review will deal with the characteristics of IRMS and GC-C-IRMS.

2. Principles of IRMS and GC–C–IRMS

2.1. IRMS

In order to understand why HRcGC is quintessential for high precision CSIA one needs to appreciate exactly how IRMS works. In contrast to so-called organic mass spectrometers (MS) that yield structural information by scanning a mass range (typically over several hundred amu) for characteristic fragment ions, IRMS instruments achieve highly precise measurement of isotopic abundance at the expense of the flexibility of scanning MS.

For isotope ratio measurement, the analyte must be converted into a simple gas, isotopically representative of the original sample, before entering the ion source of an IRMS. Continuous flow isotope ratio measurements of $^2\text{H}/^1\text{H}$, $^{15}\text{N}/^{14}\text{N}$, $^{13}\text{C}/^{12}\text{C}$, $^{18}\text{O}/^{16}\text{O}$ and $^{34}\text{S}/^{32}\text{S}$ are performed on gases of H_2 , N_2 , CO_2 , CO and SO_2 , respectively, with ^{13}C abundance measurements accounting for almost 70% of all gas isotope ratio analyses made. One also has to bear in mind that IRMS, in fact, determines the difference in isotope ratio with great precision and accuracy rather than the absolute isotope ratio. IRMS measurements yield the information of isotopic abundance of the analyte gas relative to the measured isotope ratio of a standard or reference gas. This is done to compensate for mass discriminating effects that may fluctuate with time and from instrument to instrument. In dual-inlet IRMS systems, sample gas and standard gas are introduced into two separate gas reservoirs (bellows) and a changeover valve array is used to toggle bellow effluents between the ion source and a waste line, thus maintaining constant viscous flow.

To achieve accurate and highly precise measurement of isotope ratios, obviously great care must be taken to ensure that no part of the analyte data is lost. In the case of CO_2 , the data comprise three ion traces for the different isotopomers $^{12}\text{C}^{16}\text{O}_2$, $^{13}\text{C}^{16}\text{O}_2$ and $^{12}\text{C}^{18}\text{O}^{16}\text{O}$ with their corresponding masses at m/z 44, 45 and 46, respectively. The three ion beams are registered simultaneously by a multiple Faraday cup (FC) arrangement with a dedicated FC for each isotopomer. The resulting ion currents are continuously monitored, subsequently digitised and trans-

ferred to the host computer. Here, the peak area for each isotopomer is integrated quantitatively and the corresponding ratios are calculated.

In both application areas of IRMS (low level enrichment and natural abundance work), small variations in very small amounts of the heavier isotope are detected in the presence of large amounts of the lighter isotope. The abundance A_s of the heavier isotope n_2 in a sample s , given in at.%, is defined as:

$$A_s = R_s / (1 + R_s) \times 100 \text{ (at.\%)} \quad (1)$$

where R_s is the ratio n_2/n_1 of the two isotopes for the sample. The enrichment of an isotope in a sample as compared to a standard value (A_{std}) is given in at.% excess (APE):

$$\text{APE} = A_s - A_{\text{std}} \quad (2)$$

Since the small variations of the heavier isotope habitually measured by IRMS are of the order of 0.001–0.05 at.%, the δ -notation in units of per mil (‰) has been adopted to report changes in isotopic abundance as a per mil deviation compared to a designated isotopic standard:

$$\delta_s = (R_s/R_{\text{std}} - 1) \times 1000 \text{ (‰)} \quad (3)$$

where R_s is the measured isotope ratio for the sample and R_{std} is the measured isotope ratio for the standard.

2.2. GC–C–IRMS

From the above it is obvious that a GC cannot be directly coupled to an IRMS. The need for sample conversion into simple gases has prompted the design of a combustion interface where the GC effluent is fed into a combustion reactor (Fig. 1). This reactor, either a quartz glass or ceramic tube, is filled with CuO/Pt or $\text{CuO}/\text{NiO}/\text{Pt}$ and maintained at a temperature of approximately 820 or 940°C, respectively [5,6]. The influence of combustion tube packing on analytical performance of GC–C–IRMS has been reported by Eakin et al. [7]. To remove water vapour generated during combustion, a water trap is required. Most instrument manufacturers employ a Nafion tube for this purpose. Nafion is a fluorinated polymer that acts as a semi-permeable

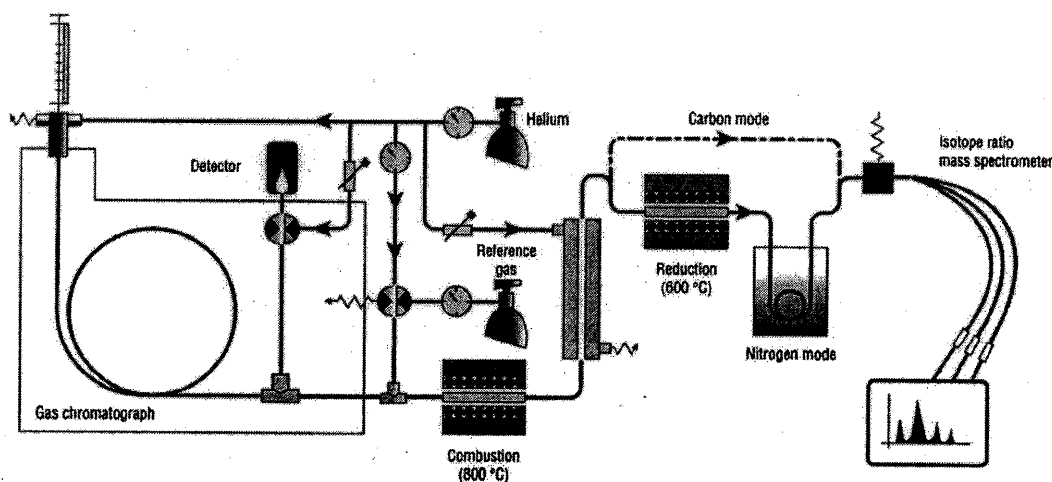


Fig. 1. Set-up of an isotope ratio mass spectrometer coupled to a gas chromatograph via a combustion interface to measure $^{13}\text{C}/^{12}\text{C}$ (carbon mode) or $^{15}\text{N}/^{14}\text{N}$ ratios (nitrogen mode). This schematic shows the reference gas set-up used for automated internal isotopic calibration [24].

membrane through which water passes freely while all the other combustion products are retained in the carrier gas stream. Quantitative water removal prior to admitting the combustion gases into the ion source is essential because any water residue would lead to protonation of CO_2 to produce HCO_2^+ , which interferes with analysis of $^{13}\text{CO}_2$ (isobaric interference). Very recently, a detailed study of this effect has been reported by Lecktrone and Hayes [8].

In dual-inlet systems, the analyte gas comes from a reservoir and only travels a short distance prior to entering the ion source. For this reason, the gas pulses result in rectangularly shaped signals. In contrast, in continuous flow IRMS (CF-IRMS) systems used for gas isotope analysis on-line gas purification steps and overall interface length lead to Gaussian-shaped signals. This is evidently even more pronounced in GC-C-IRMS systems, where analyte peaks eluting from the GC column are fed into an on-line microchemical reactor to produce, e.g., CO_2 peaks. However, due to the chromatographic isotope effect [9–11] the m/z 45 signal ($^{13}\text{CO}_2$) precedes the m/z 44 signal ($^{12}\text{CO}_2$) by 150 ms on average (Fig. 2) [5], an effect not observed in ordinary CF-IRMS systems. This time displacement depends on the nature of the compound and on chromatographic parameters such as polarity of the stationary phase, column temperature and carrier gas flow [12]. There-

fore, loss of peak data due to unsuitably set time windows for peak detection and, hence, partial peak integration will severely compromise the quality of the isotope ratio measurement by GC-C-IRMS, as will traces of peak data from another sample compound due to close proximity resulting in peak overlap with the sample peak to be analysed. Due to the fact that isotope ratios cannot be determined accurately from the partial examination of a GC peak, HRcGC resulting in true baseline separation for adjacent peaks is of paramount importance for high-precision CSIA.

It should be noted, that the chromatographic isotope effect is not caused by a vapour pressure effect but is the result of different solute/stationary phase interactions that are dominated by Van der Waals dispersion forces leading to an earlier elution of the heavier isotopomer [11]. This difference in chromatographic solute/stationary phase interaction is caused by lower molar volumes of the labelled, and thus heavier, compounds. The reason for the decrease in molar volume is the increased bond strength and thus shortened bond length between $^{13}\text{C}-\text{H}$ and, to a lesser degree, $^{12}\text{C}-^{13}\text{C}$, and $^{12}\text{C}-\text{H}$ and $^{12}\text{C}-^{12}\text{C}$, respectively. If the chromatographic isotope effect would indeed be the result of a vapour pressure effect, one would expect the lighter isotope species of a given compound to elute more rapidly

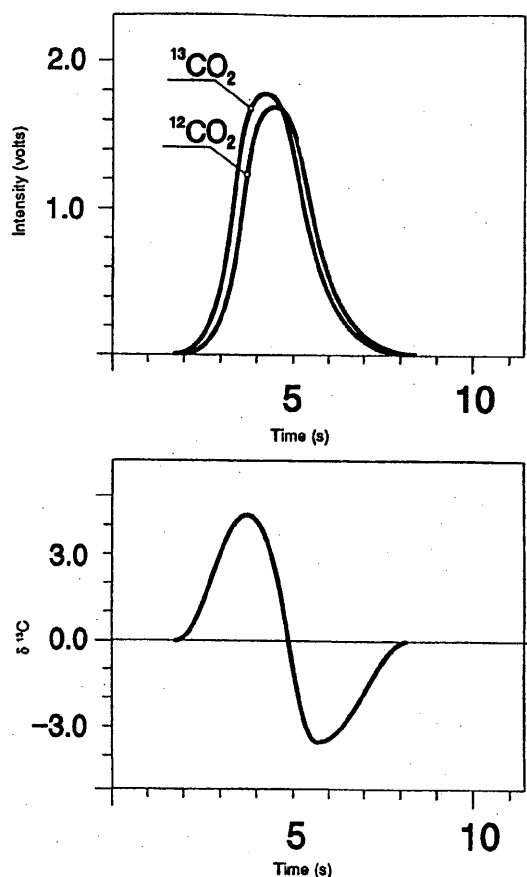


Fig. 2. Illustration of the time displacement between $^{13}\text{CO}_2$ and $^{12}\text{CO}_2$ that causes the S-shaped 45/44 ratio signal [5]. From Ref. [5], ©Intercept 1990.

from the column because of its higher vapour pressure and, hence, lower boiling point as compared to the heavier isotope species.

Although baseline separated peaks should be the ultimate goal in GC–C–IRMS, there is many an application where overlapping peaks simply cannot be avoided. In addition, CO_2 and N_2 disperse more freely within the carrier gas stream than their parent organic compounds resulting in overlapping CO_2 peaks for barely baseline resolved GC peaks. To extract the valuable information obscured by such peak overlaps, Goodman and Brenna [13,14] suggested software algorithms for improved data processing. These algorithms were based on curve fitting rather than the summation (the industrial

standard) using combinations of exponentially modified Gaussian (*E*) and Harhoff/Van-der-Linde (*H*) functions and were tested on up to 70% valley peak overlap. When the adjacent peaks were of equal abundance (leading peak:trailing peak, 1:1) combinations of HE and HH appeared to provide the best recovery of isotope ratios. In the case of unequal abundance in favour of the leading peak (10:1), the HH combination gave the best accuracy. When the abundance was reversed (1:10), the EH combination provided the best accuracy but only for peak overlap up to 40% valley. Despite these encouraging results, curve-fitting algorithms for restoring lost accuracy have not been incorporated into any commercial IRMS data reduction software by IRMS manufacturers. It could be argued that the potential of curve-fitting algorithms was only demonstrated on two compounds, methyl tridecanoate and butylated hydroxytoluene, which were of almost identical carbon isotope ratios and that any curve-fitting software should also be able to extract accurate and precise isotope ratios of two overlapping compound peaks with different carbon isotope ratios. However, any progress in this direction needs to be aided by full evaluation of new algorithms for routine use (under 'real life conditions'), thus requiring wide user access to such algorithms which in turn depends on the support from IRMS manufacturers.

2.2.1. Sample preparation

To achieve high-precision CSIA by GC–C–IRMS the following points must be considered:

- (1) Every step of the sample preparation protocol (collection, work up, derivatization) must be scrutinised for potential mass discriminatory effects to avoid isotopic fractionation of the target compounds.
- (2) If the potential of isotopic fractionation cannot be ruled out conclusively, an internal standard, of a similar chemical nature (but not requiring derivatization) and of known isotopic composition, should be added to the sample prior to sample preparation.
- (3) Signal size and isotopic composition of the standard(s) must match those of the analyte(s) [15].
- (4) The potential of all GC parameters (polarity of stationary phase, carrier gas management, temperature programme) and techniques should be exploited to their fullest to achieve HRcGC.

2.2.1.1. Derivatization

Despite their importance for high-precision CSIA, dedicated studies addressing issues of sample preparation are few and far between. Schumacher et al. compared different sample preparation methods for isotopic analysis of volatile organic compounds (VOCs) from strawberries [16]. Khalfallah et al. reported a correction method to compensate for ^{13}C tracer dilution by carbon added during derivatization [17], and a carbon balance equation was described by Demmelmair and Schmidt to calculate $\delta^{13}\text{C}$ values of free amino acids from $\delta^{13}\text{C}$ values of their derivatives at natural abundance level [18]. Kinetic isotope effects associated with derivatization reactions and resulting theoretical considerations for calculating $\delta^{13}\text{C}$ values have been discussed by Rieley [19].

Of course, one way of avoiding the problems with derivatization is not to derivatize the sample at all. This approach involves the use of moderately polar to polar stationary phases and high-temperature GC. However, not all polar compounds are amenable to these techniques (e.g., amino acids) and high-temperature capability of polar stationary phases is limited even when oxygen free helium is used as carrier gas.

In addition to changes in ^{13}C isotopic signature by derivatization, its effects on GC separation and sample conversion into CO_2 and N_2 have to be considered. Derivatization by silylation might hamper GC separation as the apolar nature of trimethylsilyl (TMS) and *tert*-butyldimethylsilyl (tBDMS) derivatives can obscure compound characteristics that could otherwise be chromatographically exploited. Furthermore, an excessive carbon load introduced by derivatization might result in incomplete combustion thus compromising accurate isotopic analysis. For reasons of non-quantitative sample conversion, the use of trifluoroacetates (TFA) or heptafluorobutyrate (HFB) is not advisable as fluorine forms extremely stable fluorides with Cu and Ni, thus irreversibly reducing combustion efficacy of the CuO/NiO system. In addition, fluorine poisons the combustion catalyst platinum. Experiments with *N*-TFA, *O*-propylates of alanine and leucine have shown that only 50% of the expected CO_2 yield was produced [20].

2.2.2. Isotopic calibration

For reasons mentioned before, it is not possible in GC–C–IRMS to calibrate target compounds against a standard of known isotopic composition, introducing the standard in exactly the same way as the analyte. There are only three feasible means of introducing a standard: (a) addition of reference compounds to the sample, (b) introduction of reference gas pulses to the carrier gas stream, or (c) introduction of reference gas pulses directly into the ion source.

Caimi et al. comprehensively listed all the desirable properties internal reference compounds should possess: (1) high chemical stability; (2) conveniently available in high purity; (3) readily soluble in high-purity solvents; (4) low vapour pressure at room temperature and atmospheric pressure; (5) environmentally rare; (6) ideally useful for GC and liquid chromatography (LC) techniques; and (7) sufficiently different chromatographic characteristics to avoid partial or complete co-elution with sample analytes [21].

The results of an extensive study into methods of isotopic calibration by Merritt et al. emphasised these demands [22]. Comparing the use of internal reference compounds with the introduction of reference gas pulses directly in the ion source of the IRMS, Merritt et al. found an offset of $>2\%$ between the two methods in the case of incomplete combustion and other systematic errors affecting only the analytes. These systematic errors affected both the analytes and the co-injected reference compounds but were not reflected by the external reference gas pulses. Similar observations were made by other groups interested in isotopic calibration [12,21,23]. In the absence of such systematic errors, Merritt et al. found that both methods of isotopic calibration gave consistent results as long as multiple reference peaks were used to permit drift correction. Only one reference peak for isotopic calibration, albeit from an internal reference compound, is not enough to compensate for the influence of GC parameters, such as analyte/stationary phase interaction, column temperature on measured isotope ratios [12].

Within the GC–C–IRMS system, seven potential sources for mass discrimination and, hence, sys-

tematic errors can be identified: (1) isotopic fractionation during sample injection (which can be overcome by on-column or time programmed splitless injection); (2) chromatographic isotope effect; (3) chromatographic peak distortion (leading and trailing peak tail); (4) combustion process; (5) peak distortion of N_2/CO_2 gas peak during passage of the combustion interface; (6) changing flow conditions at the open split prior to the IRMS; and (7) the IRMS itself. Obviously, the external reference gas pulses only compensate for item (7), whereas internal reference compounds reflect all of the aforementioned. Recently, a method for isotopic calibration was reported that, provided a combustible gas was used, could reflect the systematic errors caused by items (4–7) [24]. This method combines the convenience and practicability of external reference gas calibration with the advantage of reflecting the majority of physical influences to which analytes are subjected in a GC–C–IRMS system.

2.2.3. HRcGC

As pointed out earlier, baseline separated gas chromatographic peaks are the basis for high-precision CSIA. To achieve this goal, in the first instance, basic gas chromatographic rules must be observed: (1) the polarity of the stationary phase should meet the polarity of the analytes; (2) column head pressure and, hence, carrier gas velocity, should be set to suit column diameter; and (3) temperature gradients should be chosen to exploit the maximum of the

column length (the longer the column, the slower the temperature rise per minute; cf. Table 1) [25].

Further to these principles, HRcGC techniques such as multi-dimensional capillary GC (MDcGC), enantio-selective GC, porous layer open tubular (PLOT) column GC for analysis of VOCs and high-temperature capillary GC (HTcGC) are powerful tools for high-precision CSIA when used in combination with GC–C–IRMS. Nitz et al. were the first to report the advantages of using MDcGC in GC–C–IRMS [26]. MDcGC is now, often in combination with enantioselective GC, almost exclusively used in authenticity control of flavours and fragrances by CSIA [27,28]. In a similar fashion, HTcGC is strongly associated with CSIA of steroids and long-chain fatty acids (e.g., Ref. [29]).

Regrettably, the achievements of HRcGC in terms of well-defined peak shape and baseline separation are likely to be impaired during combustion and the subsequent passage through the interface. Changes in tubing diameter and frequent use of unions to connect the various parts of tubing lead to a loss in peak definition (peak broadening; peak distortion) and even to partial peak overlap, all of which have a detrimental effect on accuracy and precision of isotope ratio measurement [12]. Very recently, Goodman reported a single-capillary interface design (SCID) which he developed to overcome these problems [30]. As the name suggests, a single capillary was used to connect the GC column to the open-split in front of the IRMS. This capillary was threaded through a furnace and accommodated two

Table 1

Recommended values for carrier gas velocity and temperature gradient according to column length when using helium as carrier gas^a

| Column length (m) | Elution of methane (s) ^b | Temperature gradient (°C/min) |
|-------------------|-------------------------------------|-------------------------------|
| 10 | 35 | 2.5 |
| 15 | 53 | 1.65 |
| 20 | 70 | 1.25 |
| 25 | 88 | 1.05 |
| 30 | 105 | 0.84 |
| 40 | 140 | 0.63 |
| 50 | 175 | 0.5 |

^aBased on working directions given by Grob [25].

^bSet GC oven temperature to 30°C. Set split ratio to about 1:30, inject a few μ l of natural gas (or lighter gas) and measure elution time of the first peak (FID signal). Adjust column head pressure to match recommended elution time.

CuO wires positioned thus as to coincide with the furnace dimensions. So far, this design has been tested for ^{13}C isotopic abundance analysis of *n*-alkanes.

3. High-precision CSIA of ^{13}C isotopic abundance

3.1. GC–C–IRMS at natural abundance level

High-precision CSIA of ^{13}C isotopic abundance at both natural abundance (NA) and low enrichment

level can yield measurements of $\delta^{13}\text{C}$ values with a precision of 0.3‰ on average. Thanks to this high precision, even small changes in ^{13}C isotopic abundance of 1‰ can be reliably detected. For this reason GC–C–IRMS has become the method of choice to determine the origin of a given organic compound by measuring its characteristic isotope ‘finger print’.

In contrast to the generally held opinion, the natural abundance of stable isotopes is not a fixed constant but displays a considerable, yet subtle, degree of variation. The variation on the natural abundance of ^{13}C can be as high as 0.1 at.% (Fig. 3). This wide range reflects the varying degree of mass

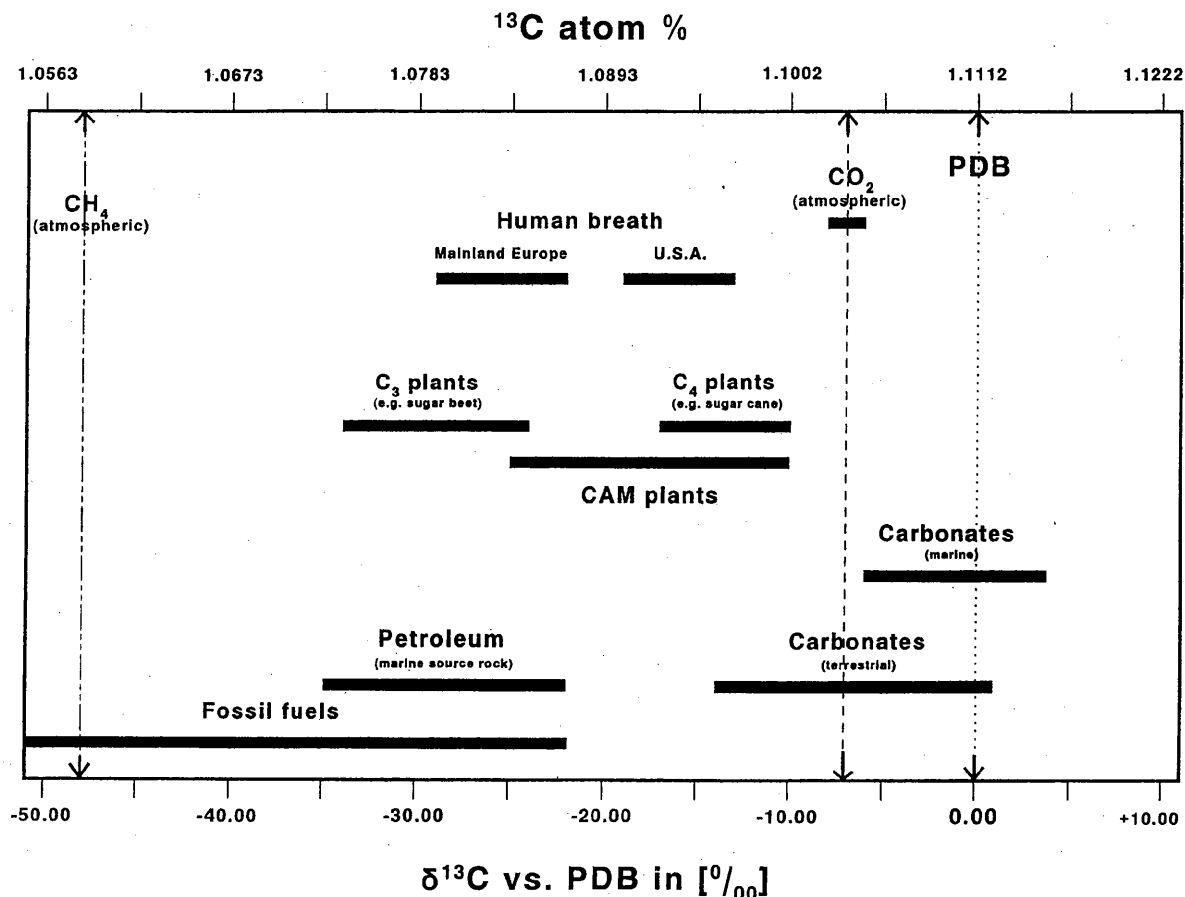


Fig. 3. Some typical examples of natural $\delta^{13}\text{C}$ values grouped according to origin along the scale of ^{13}C natural abundance.

discrimination associated with the different pathways of carbon assimilation and fixation. To give an example, in terms of ^{13}C isotopic abundance, beet sugar is not the same as cane sugar. In sugar beet CO_2 fixation results in the formation of a C_3 body, 3-phosphoglycerate (3-PGA). This pathway of CO_2 fixation is known as the Calvin cycle. Plants using the 3-PGA pathway for CO_2 fixation are commonly called C_3 plants. However, some plants, of which sugar cane is one, make use of a different pathway. Here, CO_2 fixation yields a C_4 -dicarboxylic acid, oxalo acetate, hence the term C_4 plants (the C_4 -dicarboxylic acid pathway is also known as the Hatch–Slack cycle). The products of these two pathways are characterised by their different ^{13}C abundance. Glucose derived from C_3 plants has a $\delta^{13}\text{C}$ value of about -25‰ , whereas glucose derived from C_4 plants exhibits a more positive $\delta^{13}\text{C}$ value of about -11‰ indicating a mass discriminatory bias towards $^{13}\text{CO}_2$ of the Hatch–Slack cycle.

Differences in $\delta^{13}\text{C}$ values were reported for total leaf tissue, total surface lipid extracts, and individual n -alkanes isolated from plants utilising either C_3 , C_4 , and crassulacean acid metabolism (CAM) pathways for carbon fixation [31,32]. The average $\delta^{13}\text{C}$ values obtained from C_3 plant material were between 10 and 15‰ lower compared to the corresponding $\delta^{13}\text{C}$ values obtained from C_4 and CAM plant material. Measurement of the isotopic abundance of ^{13}C for lutein isolated from marigold (C_3) and maize (C_4) yielded -29.90 ± 0.20 and $-19.77 \pm 0.27\text{‰}$, respectively, showing a similar difference of $>10\text{‰}$ between the two photosynthetic pathways [33].

Although the vast majority of plants belong the C_3 group ($>300\,000$) with bulk $\delta^{13}\text{C}$ values of $<-24\text{‰}$, differences in the rate of photosynthesis (mainly caused by differences in climate and geographical location) and in enzyme kinetics of biochemical pathways result in subtle variation of $^{13}\text{C}/^{12}\text{C}$ ratios that can be detected by GC–C–IRMS. Lockheart et al. were able to detect inter- and intra-specific differences in $\delta^{13}\text{C}$ values for n -alkanes and alcohols in sun and shade leaves from oak and beech ranging from 0.7 to 3.0‰ [34]. These subtle differences in $\delta^{13}\text{C}$ values can be used to determine origin of an organic compound and, thus, the authenticity of a sample.

3.1.1. Authenticity

3.1.1.1. Flavours and fragrances

Bernreuther et al. measured $^{13}\text{C}/^{12}\text{C}$ isotope ratios of natural and nature-identical γ -decalactone and reported significant differences in $\delta^{13}\text{C}$ values that were source dependent although all plants belonged to the C_3 group [35]. For natural γ -decalactone from stone fruit (apricot and peach) they found $\delta^{13}\text{C}$ values ranging from -38.0 to -40.8‰ , respectively, that were significantly different from $\delta^{13}\text{C}$ values of γ -decalactone extracted from soft fruit (strawberry) which were -29.2‰ on average. In contrast, $\delta^{13}\text{C}$ values of artificial (nature-identical) γ -decalactone ranged from -24.4 to -26.9‰ , whereas γ -decalactone of biotechnological origin showed $\delta^{13}\text{C}$ values of -30.8‰ on average.

In an independent study, Mosandl et al. demonstrated the additional advantage to be gained from enantioselective GC–C–IRMS on samples of synthetic γ -decalactone (racemate RAC; 4R:4S = 50:50), γ -decalactone of biotechnological origin (BIO; 4R $> 99\%$ ee), and a mixture of both (BIO: RAC = 60:40) [36]. They reported for the enantiomerically pure (4R)- γ -decalactone of biotechnological origin a $\delta^{13}\text{C}$ value of $-30.12 \pm 0.14\text{‰}$ which is in good agreement with the $\delta^{13}\text{C}$ values measured by Bernreuther et al. The separated enantiomers of the nature-identical product showed, as could be expected, identical $\delta^{13}\text{C}$ values within the observed standard deviation (4R, $-28.32 \pm 0.36\text{‰}$; 4S, $-28.18 \pm 0.39\text{‰}$). Mixing 60% of BIO with 40% of RAC yielded an enantiomeric distribution of 4R:4S = 80:20, with 75% of the 4R-configured γ -decalactone being of biotechnological origin. The measured $\delta^{13}\text{C}$ value of the 4R enantiomer in this mixture was $-29.62 \pm 0.33\text{‰}$ which was in good agreement with the theoretically expected value of -29.67‰ . The $\delta^{13}\text{C}$ value of the 4S enantiomer in this mixture was of course the same as for the 4S enantiomer in the pure synthetic sample ($-28.08 \pm 1.30\text{‰}$).

This early work suggested that two phenomena of biosynthetic pathways, enantioselectivity and mass discrimination (kinetic isotope effects), might serve as compound-specific parameters to establish origin and, hence, to control authenticity of natural flavours and fragrances. However, when focused on indi-

vidual compounds the application of stable isotope abundance measurement is only of limited use as most plants cultivated for human consumption are C_3 plants whose ^{13}C isotopic signatures partially overlap with those of synthetic compounds derived from fossil sources or those of compounds produced by biotechnological methods.

This problem was soon recognised and Mosandl's group suggested the use of genuine internal isotopic standards and formulated the following recommendations [37,38]:

- (1) the compound selected as internal isotopic standard should be a genuine characteristic compound of lesser sensorial relevance;

- (2) the compound must be available in the sample in sufficient amounts and must not be susceptible to mass discrimination during sample preparation;

- (3) the selected compound must be biogenetically related to the compounds under investigation;

- (4) chemical inertness of the compound during storage and/or technical processes is mandatory;

- (5) the compound selected as internal isotopic standard must not be a legally allowed additive.

The obvious advantage of using an internal standard, biogenetically related to characteristic sample compounds, is the elimination of variations in the $\delta^{13}C$ -signature resulting from, e.g., climate-dependent variations of the photosynthetic rate and the kinetic isotope effect associated with the CO_2 fixation step. Therefore, only the characteristic kinetic isotope effects caused by enzymatic reactions during secondary biogenetic pathways are investigated, and the resulting relative $\delta^{13}C$ values can be used as a sample-specific finger print (cf. Fig. 4).

Applying the aforementioned recommendations, Mosandl and co-workers, who have to be regarded as the leaders in the field of authenticity control, studied a wide range of commercially relevant flavours and fragrances and assessed authenticity of allegedly natural samples from commercial sources. The materials studied included lemon oil [37], balm oil [39], citronella oil [39], lemongrass oil [39], coriander oil [40], bergamot oil [41], orange oil [42], mandarin oil [43,44], peppermint oil [45], volatile components from strawberries [16] and apples [46] and α - as well as β -ionone from raspberries [47]. Using self-prepared authentic samples, sample-specific finger prints of six to eight biogenetically related compounds

were established based on $\delta^{13}C$ values relative to an internal standard (either limonene, γ -terpinene or neryl acetate). With the help of these finger prints, samples made up entirely of synthetic compounds and even samples containing mixtures of authentic (natural) material and synthetic compounds could be reliably identified. In cases where chiral compounds such as linalol occur naturally as enantiomeric mixtures (in coriander oil, $R:S=20:80$), enantioselective GC-C-IRMS could even detect non-authentic samples imitating the natural enantiomeric ratio by measuring the $\delta^{13}C$ values of each enantiomer [40,48].

3.1.1.2. Wine, fruit juice and honey

In the early days of GC-C-IRMS, detection of added sugar in fruit juice and wine was fairly simple as mainly cheap corn syrup (maize: C_4 plant) was predominantly used to boost sugar and/or ethanol content, respectively. Measuring $\delta^{13}C$ values of glucose or bulk carbon was sufficient to prove adulteration. However, addition of small amounts of C_4 plant sugars ($\leq 10\%$) to C_3 plant products such as wine, fruit juice and honey, or the addition of sugars from other C_3 plants (sugar beet; concentrated and decaffeinated grape juice) could no longer be detected by these measurements [49]. Schmidt and co-workers studied several methods based on high-precision CSIA to determine authenticity of wine, fruit juices and honey, and to prove fraudulent addition of sugars and even vitamin C from other sources. Their research revealed that in authentic fruit juices biogenetically related compounds such as L-ascorbic acid [50,51], L-malic acid [52] and L-tartaric acid [51] showed $\delta^{13}C$ values strongly correlating with those of their corresponding sugars. For instance, the $\delta^{13}C$ value of L-ascorbic acid is +4.8‰ higher than that of its precursor glucose. This enrichment is mainly located in the C-1 position of L-ascorbic acid of authentic origin and the result of plant-specific kinetic isotope effects during biosynthesis, whereas L-ascorbic acid of biotechnological origin preserves the ^{13}C pattern of glucose [51].

In glycerol originating from natural sources, Weber et al. found a ^{13}C depletion position specific for C-1 and they suggested this unique feature might be used as a means to test for illegal addition of synthetic glycerol to wines [53]. They also discov-

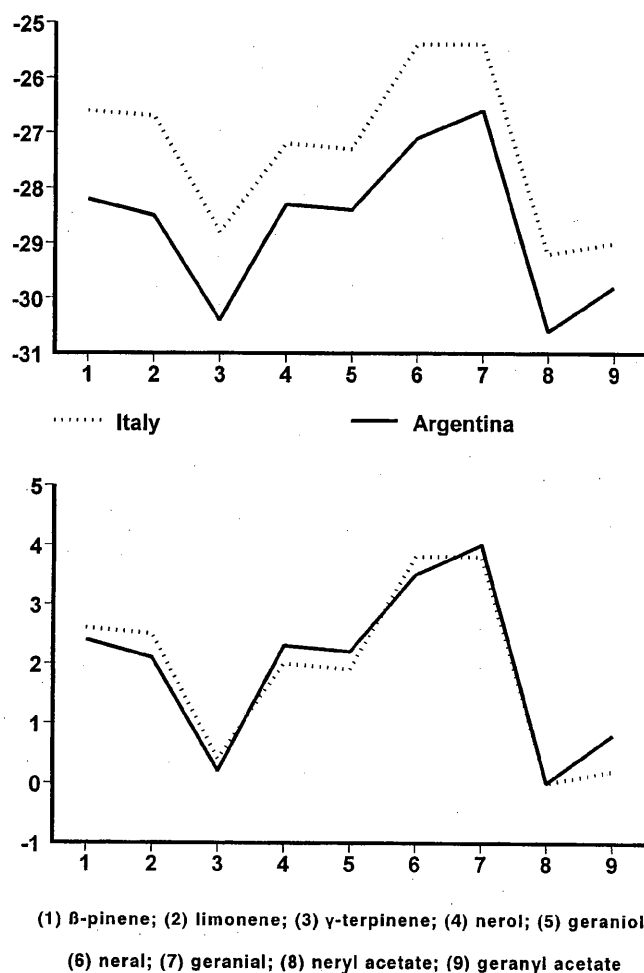


Fig. 4. $\delta^{13}\text{C}$ fingerprint of biogenetically related compounds in lemon oils of different geographical origin (top graph). The graph at the bottom shows the $\Delta\delta^{13}\text{C}$ fingerprint of the samples obtained when using neryl acetate (8) as internal isotopic standard [38]. From [38], ©Marcel Dekker, 1995.

ered a constant $\Delta\delta^{13}\text{C}$ correlation between ethanol and citric acid (+2.4‰) in addition to the known $\Delta\delta^{13}\text{C}$ correlation between fermented sugar and ethanol (−1.7‰) [54]. Dennis et al. suggested the use of $\delta^{13}\text{C}$ values of sorbitol as a further means for authenticity control of wines [55].

3.1.1.3. Vegetable oils

High-quality, single-source vegetable oils are another target for fraudulent adulteration, i.e., partial or total substitution of minor quality and, hence,

cheaper oils for the high quality product. In a blind study, Woodbury et al. were able to detect the adulteration of maize germ oil with oils of C_3 plant origin down to a level of 5% (w/w) [56]. They found the saturated 16:0 fatty acid in maize oil to be more depleted in ^{13}C than the corresponding unsaturated fatty acids 18:1 and 18:2. In addition, consistent differences were observed for $\delta^{13}\text{C}$ values of vegetable oils from different geographical regions. In a subsequent study, Woodbury et al. determined fatty acid composition and $\delta^{13}\text{C}$ values of the major fatty acids of more than 150 vegetable oils [57], thus

establishing a database that provides isotopic information for authenticity control of vegetable oils. Variability in $\delta^{13}\text{C}$ values could be related to geographical origin, year of harvest, and the particular variety of oil. Their findings suggest that ultimately $\delta^{13}\text{C}$ values of fatty acids are determined by a combination of environmental and genetic factors.

Kelly et al. investigated authenticity of single-seed vegetable oils of C_3 plant origin such as groundnut, palm, rape seed and sunflower oils [58]. They found that the $\delta^{13}\text{C}$ values for the authentic vegetable oil fatty acids fell within a narrow range of -27.6 to -32.1‰ . Employing canonical discriminant analysis, ^{13}C data from sunflower oil could be separated from other oils, exploiting small, yet significant, differences in $\delta^{13}\text{C}$ values within the oil varieties. To detect adulteration of olive oils, Angerosa et al. compared $\delta^{13}\text{C}$ values of the aliphatic alcoholic oil fractions and found those of the adulterant pomace oil to be significantly more negative than those of virgin and refined olive oils [59]. Furthermore, they studied isoprenoids and methylsterols isolated from each grade of olive oil and showed the better the olive oil grade, the more positive (i.e., less negative) the $\delta^{13}\text{C}$ values of these compounds became.

3.1.1.4. Drugs

Measuring ^{13}C isotopic abundance of heroin to trace the origin of heroin samples in narcotic drug abuse, showed some evidence of variation in $\delta^{13}\text{C}$ values of heroin depending on its geographical site of production [60]. A preliminary study to trace the origin of different batches of confiscated 3,4-(methyldioxy)methylamphetamine (MDMA, Ecstasy) tablets by GC–C–IRMS allowed the discrimination of four different groups of MDMA tablets based on variations in their NA $\delta^{13}\text{C}$ values [61]. The same study showed that further discrimination could be obtained when using $\delta^{15}\text{N}$ values of MDMA.

Prompted by the uncertainty associated with the $\text{T/E} > 6$ test that measures the ratio of testosterone (T) and epitestosterone (E) and the high public interest in alleged doping in athletes, several studies have been carried out to use metabolic pathway related ^{13}C isotope patterns to differentiate between endogenous human testosterone and exogenous testosterone [62]. In the human body, testosterone is

synthesised from cholesterol via dehydroepiandrosterone and is then further metabolised to androstenediol. The group around Aguilera et al. found that the averaged $\delta^{13}\text{C}$ values for endogenous 5α - and 5β -androstenediol dropped from -26.52‰ before synthetic testosterone administration (natural background or baseline value) to -32.44‰ during testosterone administration [63]. Independent work by Shackleton et al. obtained a similar baseline $\delta^{13}\text{C}$ value of -26.87‰ and a drop down to -30.21‰ on average for androstenediol samples after a bolus administration of 250 mg exogenous testosterone [64,65]. This drop of about -2‰ lasted for up to 10 days before $\delta^{13}\text{C}$ values of androstenediol returned to their former baseline value. They also reported a narrow range of -29.15 to -30.41‰ for five synthetic testosterone samples manufactured in five different countries. Based on the results reported by Aguilera et al. [63] and their own, Shackleton et al. suggested a conservative cut-off value of -29.0‰ for androstenediol to identify unambiguously testosterone abuse for up to 7 days after administration.

Hydrocortisone abuse in horse racing and other equine sports can be confirmed on the basis of significantly different ^{13}C isotope patterns between endogenous urinary hydrocortisone and synthetic material. This method, proposed by Aguilera et al., employs conversion of urinary hydrocortisone into its bismethylenedioxy derivative to improve its gas chromatographic properties [66].

3.1.2. Origin

3.1.2.1. Geochemistry

The desire to study ^{13}C isotope abundance of sedimentary hydrocarbons on a molecular level was one of the driving forces behind the development of GC–C–IRMS. Geochemists and archaeologists wanted to extract all possible information contained in fossil biomarkers such as sedimentary long-chain alcohols and sterols [67], triterpene-derived hydrocarbons [68], neutral monosaccharides [69], long-chain alkanes [70–74], alkanes and isoprenoids [75,76], polycyclic aromatic hydrocarbons (PAHs) [77], amino acids [78–80] and phenolic acids [81].

Freeman et al. measured hydrocarbons from sediments deposited in the Messel shale and found a wide spectrum of $\delta^{13}\text{C}$ values ranging from -20

down to -75% , thus proving the equally wide spectrum of origins [82]. The extreme negative values were thought to indicate the activity of methanotrophic bacteria, as $\delta^{13}\text{C}$ values of $<-45\%$ thus far had only been observed for methane but not for larger molecules. This and others studies [68,69,73,83] demonstrated that sedimentary organic compounds contain contributions of bacterial origin rather than being solely of plant origin. Using GC–C–IRMS to measure ^{13}C isotope abundance in hydrocarbons from sedimentary rocks across the Precambrian–Cambrian boundary, Logan et al. [84] were able to shed some light on the development of multicellular life during the so-called ‘Cambrian explosion’. Based on the isotopic data, they could show a transition from an environment dominated by sulphate-reducing bacteria to one dominated by photosynthetic organisms, thus transforming the hitherto anaerobic ocean into an aerobic ocean.

3.1.2.2. Archaeology

Measuring $^{13}\text{C}/^{12}\text{C}$ isotope ratios has become an increasingly important tool to glean information on prehistoric diet and lifestyle from organic residues preserved in archaeological artefacts. Employing both HTcGC–MS and HTcGC–C–IRMS to identify chemical structures and measure $\delta^{13}\text{C}$ values, respectively, Evershed et al. showed that lipid extracts (C_{25} – C_{33} alkanes and a C_{29} ketone, nonacosain-15-one) from organic residues found in archaeological potsheds were derived from *Brassica* species (wild-type cabbage) [85]. Later work on organic residues from archaeological pottery vessels found C_{31} , C_{33} and C_{35} ketones with $\delta^{13}\text{C}$ values that were up to 10% higher than those found for the C_{29} ketones from wild-type *Brassica* species. Based on HTcGC–MS and HTcGC–C–IRMS data, Evershed and co-workers formed the hypothesis that a precursor/product relationship may exist between C_{35} ketones and fatty acids, and corresponding triacylglycerols such as tripalmitin and tristearin from animal fats [86]. Studying pyrolysis reactions of acyl lipids and monitoring their products by HTcGC–MS and HTcGC–C–IRMS, they could confirm that C_{31} , C_{33} and C_{35} mid-chain ketones found in archaeological pottery vessels were indeed derived from a mixture of free fatty acids [87]. Comparing carbon number distributions of triacylglycerols and $\delta^{13}\text{C}$ values of

16:0 and 18:0 acyl moieties of lipid compounds from extracts of neolithic vessels with modern reference animal fats, Evershed et al. could identify animal fat residues found in vessels dated circa 4200 BP as being close to reference pig adipose fats, whereas residues found in vessels dated circa 4500 BP were closer to reference ruminant fat [88].

O'Donoghue et al. reported $\delta^{13}\text{C}$ values in the range of -25.4 to -29.2% for the principal fatty acids (16:0 to 24:1) of radish seed found in a 6th century AD storage vessel [89]. Composition and $\delta^{13}\text{C}$ values of fatty acids found in the ancient radish seeds matched closely those found in modern radish seeds.

Stott et al. measured $\delta^{13}\text{C}$ values of cholesterol and 3β -hydroxycholest-5-en-7-one from fossil whale bones [90] and archaeological human bones and teeth [91], and showed that their ^{13}C content could be used as an important new source of palaeodietary information. Another insight into prehistoric life was gleaned from the ^{13}C analysis of adsorbed lipids preserved in the fabric of Minoan lamps and conical cups. The $\delta^{13}\text{C}$ values together with HTcGC–MS profiles identified beeswax as the illuminant burned in prehistoric Aegean lamps rather than olive oil as hitherto supposed [92].

3.1.2.3. Environmental chemistry

Monitoring environmental and climate changes by measuring $\delta^{13}\text{C}$ values of atmospheric gases such as methane, carbon monoxide and carbon dioxide has traditionally been carried out using dual-inlet IRMS systems. Their measurements however, required time-consuming sample preparation of large sample volumes. The high abundance sensitivity of GC–C–IRMS together with the use of PLOT fused-silica capillary columns for routine GC analysis of highly volatile organic compounds (HVOCs) has now become the method of choice for scientists as air samples between $50\ \mu\text{l}$ and $5\ \text{ml}$ can be analysed on-line without any prior sample preparation [93–95].

High-precision CSIA by GC–C–IRMS is also used to determine origin and identify sources of oil spills and oil pollution [96–98], ocean-transported bitumen [99], characterisation of refractory wastes at heavy-oil contaminated sites [100,101] and to trace

the sources of PAHs in the environment [102–104]. Bird et al. used GC–C–IRMS to record the $\delta^{13}\text{C}$ values of individual biomarker compounds (*n*-alkanes) to assess vegetation changes [105], whereas Naraoka et al. measured differences in $\delta^{13}\text{C}$ values of long-chain fatty acids (C_{20} – C_{30}) that had been extracted from terrestrial and marine sediment [106].

3.2. Tracer studies

The high abundance sensitivity of GC–C–IRMS has also been increasingly exploited in metabolic studies investigating turnover, incorporation and synthesis processes *in vivo* that could previously only be investigated either with stable isotope tracers at high enrichment level using GC–MS, or not at all. The latter usually for reasons of excessive tracer dilution in the various metabolic pools and/or low incorporation rates. Although in these cases radioactive tracers can provide an alternative, their use is associated with certain risks which are nowadays regarded as unacceptable especially when dealing with members of the paediatric age group.

GC–C–IRMS proved to be a decisive tool in determining if and to what extent neonates and premature infants of very low birth weight could synthesise arachidonic acid, which is essential for their growing tissues, from dietary fatty acids. Demelmair et al. could show that in neonates fed on a phenylalanine-free diet, on average 23% of free plasma arachidonic acid on study day 4 originated from infantile linoleic acid conversion [107]. They determined ^{13}C content of linoleic acid and arachidonic acid in 0.25–0.5 ml serum before and for 4 days after the infants' diet contained corn oil. Baseline $\delta^{13}\text{C}$ values for linoleic acid and arachidonic acid were -31.5 ± 1.1 and $-30.1 \pm 1.2\text{‰}$, respectively; after 4 days, changes in $\delta^{13}\text{C}$ values over baseline were $+12.7 \pm 0.7$ and $+2.7 \pm 0.7\text{‰}$, respectively. Carnielli et al. added linoleic acid and linolenic acid, both ^{13}C -labelled, to the formula diet which was administered continuously for 48 h (birth weight, 1.17 ± 0.12 kg; gestational age, 28.4 ± 1.3 weeks) [108]. They could show that both tracers were rapidly incorporated into plasma phospholipids and that their metabolic products including arachidonic acid and docosahexaenoic acid became highly

enriched with ^{13}C . Incorporation of ^{13}C -octanoic acid into plasma triglycerides (10% of the enrichment of the diet), noticeably into myristic and palmitic acid, by very low-weight preterm infants was reported earlier by the same group [109].

A similar observation was made during a study with an entirely different objective. Using GC–C–IRMS, Koziat et al. could demonstrate that ethanol itself may be used as a substrate for lipogenesis, although only to a small extent [110]. They calculated that $<10\%$ of fatty acids contained in very-low-density lipoproteins (VLDL) triglycerides were derived from this pathway, with ethanol predominantly being incorporated into myristic and palmitic acid.

The majority of ^{13}C tracer studies published have dealt with various aspects of fatty acid biochemistry, such as metabolism of triglycerides [111,112], and transport and turnover of free saturated [113–115] and unsaturated fatty acids [116–120]. While studying the metabolism of ^{13}C -labelled polyunsaturated fatty acids by ^{13}C NMR, using GC–C–IRMS, Cunnane et al. found low levels of ^{13}C -labelled γ -linolenic acid in brain phospholipids of suckling rat pups that could not be detected by ^{13}C NMR [121].

Employing ^{13}C tracers at low levels of enrichment, reliable data were obtained in studies of the kinetics of glucose [122,123] and glycoprotein neutral sugars [124,125], cholesterol and lipoproteins [126], phosphatidylcholine [127], urea [128,129], branched-chain amino acid metabolism [130], measuring protein fractional synthetic rates [131] and protein synthesis in colorectal cancer cells [132,133]. Roscher et al. used L-rhamnose with two different levels of ^{13}C abundance in parallel experiments to determine if L-rhamnose serves as a carbon source for furaneol [134].

Unlike GC–MS, where increasing the amount of label has no general effect on detection limits, in GC–C–IRMS, increasing label enrichment in precursor compounds produces significantly improved detection limits. In a recent review of high-precision CF–IRMS, Brenna et al. discussed theoretical and practical considerations for this type of study [135]. High-precision CSIA in this type of tracer studies has been shown to possess advantages over organic GC–MS for stable isotopic tracer detection and to be

superior to radio-isotopic tracer methods in terms of dose size and analysis efficiency [136]. Guo et al. reported that GC–C–IRMS provides 15-fold lower detection limits for [$^{13}\text{C}_2\text{--C3,C4}$]cholesterol than organic GC–MS [137]. Using [$\text{U-}^{13}\text{C}$]α-linolenic acid, Sheaff et al. were able demonstrate that conversion of α-linolenate into docosahexaenoate was not depressed by high dietary levels of linoleic acid [117]. An interconversion of saturated dietary fatty acids (e.g., 18:0) into unsaturated fatty acids (e.g., 18:1) in plasma of about 14% was reported by Rhee et al. [138]. Menand et al. applied this technique to measure carbon incorporation into plasma glutamine [139]. Practical aspects of this technique have been investigated by Dube et al. [140,141].

4. Compound-specific isotope analysis of ^{15}N isotopic abundance

High precision CSIA of ^{15}N isotopic abundance faces three major challenges. First, the relatively low concentration of nitrogen in organic compounds (for instance, amino acids contain two to 11 times more carbon than nitrogen). Secondly, $^{15}\text{N}/^{14}\text{N}$ isotope ratios are measured on N_2 , which means the compound conversion step requires a reduction process and, in the case of amino acids, the formation of one mol equivalent of N_2 produces 4–22 molequivalents of CO_2 (if we regard the underivatised case). Lastly, even small amounts of atmospheric gas leaks into the instrument result in a high N_2 background.

Due to the wide spectrum of applications that would benefit from a system capable of analysing ^{15}N isotopic enrichment down to natural abundance level, especially because there is no radioactive alternative, these difficulties did not delay instrument development for too long. The desire to study for example nitrogen fixation in plants and micro-organisms and nitrogen metabolism in humans, as well as the possibility to quantify gene expression by measuring mRNA turnover, prompted two groups to extend the scope of GC–C–IRMS. In 1994, Preston and Slater presented a system with a conversion interface comprising combustion furnace, liquid N_2 cold trap, to trap CO_2 and water, and a PLOT column to resolve N_2 from any CO formed by poor combustion [142]. If CO were permitted to enter the

ion source simultaneously with N_2 , this would result in a serious isobaric interference at m/z 28. For $\delta^{15}\text{N}$ values of $t\text{BDMS}$ derivatives of amino acids, they reported a precision of S.D. ($\delta^{15}\text{N}$) = 5‰ at natural abundance level.

In the same year, Merritt and Hayes presented a similar system but for the addition of a reduction furnace, loaded with Cu wires and maintained at a temperature of 600°C, to reduce N-oxides to N_2 and to scavenge O_2 emanating from the combustion furnace [143]. Their system also included a cryogenic trap to remove CO_2 and water, and it produced a precision of S.D. ($\delta^{15}\text{N}$) = 0.2‰.

This marked difference in precision was attributed to different performances of the IRMS systems. Ongoing investigations in our laboratory seem to indicate that this difference might be caused by the simultaneous presence of NO and N_2 in the ion source. Placing a PORAPLOT Q capillary column of 0.32 mm internal diameter, maintained at 30°C between the water trap and the ion source, separates N_2 from NO (Fig. 5). Comparing precision for $\delta^{15}\text{N}$ analysis of identical aliquots from the same sample with and without the PLOT column in place, we found a drop in precision from S.D. ($\delta^{15}\text{N}$) = 0.15‰ to S.D. ($\delta^{15}\text{N}$) = 2‰, respectively. This phenomenon cannot be explained by isobaric interference of NO (m/z 30) with m/z 28 ($^{14}\text{N}_2$) or m/z 29 ($^{15}\text{N}^{14}\text{N}$) but might be caused by reaction of NO with N_2 in the ion source.

To avoid potential isotopic fractionation due to various degrees of nitrogen silylation when TMS derivatives were formed, Hoffmann et al. prepared $t\text{BDMS}$ derivatives of amino acids from wheat protein hydrolysate [144]. The same strategy was followed by Segschneider et al. when investigating ^{15}N uptake from ^{15}N -labelled NO_2 by measuring $\delta^{15}\text{N}$ values of soluble amino acids in sunflower leaves [145]. However, when preparing $t\text{BDMS}$ derivatives, one has to bear in mind that in the case of, e.g., L-leucine, one adds 12 mol equivalent of carbon to 1 mol equivalent of amino acid, thus resulting in 36 mol equivalent of carbon for 1 mol equivalent of N_2 , which might give rise to CO formation due to poor combustion. Metges et al. reported an alternative derivatisation protocol for amino acids yielding *N*-pivaloyl, *O*-isopropylates, and demonstrated their application by measuring NA

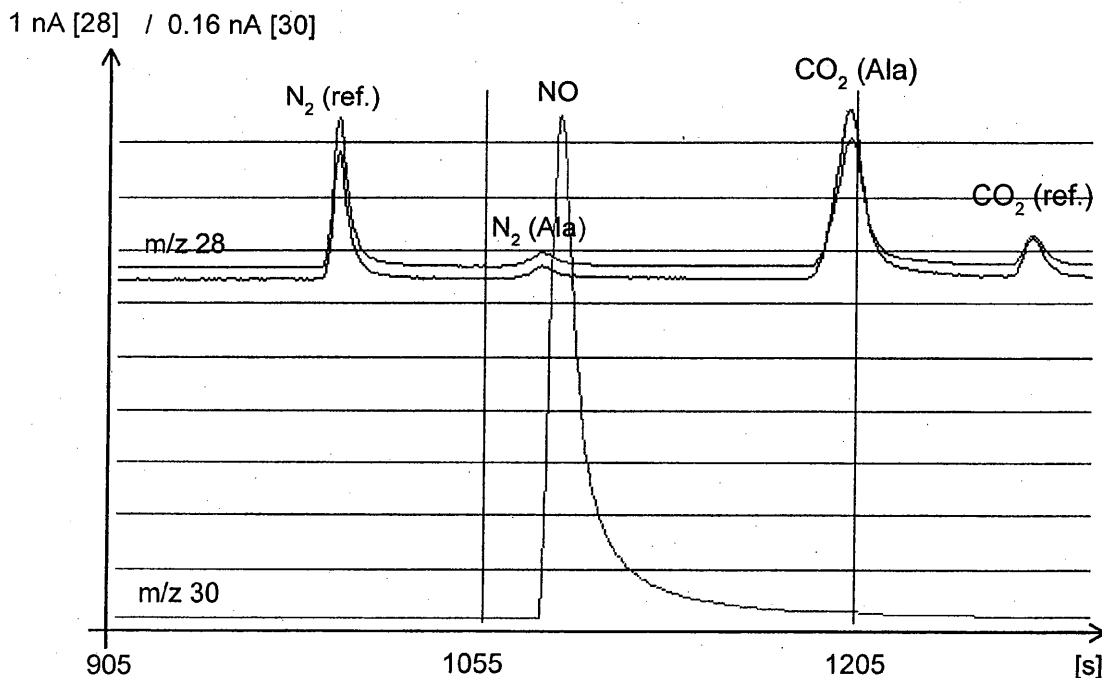


Fig. 5. The N_2^+ mass traces obtained for alanine (as *N*-acetyl, *O*-propyl derivative) having passed post-combustion through a PORAPLOT Q column, held at 30°C, shows the presence of NO next to N_2 . The CO_2 peaks are caused by the formation of CO^+ from CO_2 in the ion source.

$\delta^{15}N$ values of amino acids from plasma albumin hydrolysate [146].

CSIA of ^{15}N isotopic abundance by GC–C–IRMS has been applied to measure the effect of ibuprofen on protein synthesis using ^{15}N -labelled glycine as tracer [147]. Williams et al. used ^{15}N -labelled urea to demonstrate that *Helicobacter pylori* uses urea as a nitrogen source for its synthesis of amino acids [148]. A multidisciplinary group comprising environmental, archaeological, geological and nutritional scientists showed that differences in $\delta^{15}N$ values from soil amino acids could be used to indicate differences in land use in Bronze Age, medieval and early modern soils [149]. One intriguing observation they made was the consistently low levels of ^{15}N abundance ($\delta^{15}N < 0.0\%$ vs air) in the amino acids threonine (Thr) and phenylalanine (Phe) from soils of unmanured cereal production sites, whereas $\delta^{15}N$ values of all the other amino acids were positive. We thought this was intriguing because a similar pattern was found by Metges and Petelec when monitoring

$\delta^{15}N$ values of free plasma amino acids from fasting human subjects. With the exception of Thr and Phe, all other amino acids showed positive $\delta^{15}N$ values [150].

Mas et al. suggested that $\delta^{15}N$ values of MDMA (ecstasy) could be used for batch discrimination of ecstasy tablets [61], and Faulhaber et al. used $\delta^{15}N$ values of methyl-*N*-methylantranilate as a biomarker in the authenticity control of mandarin oils [43]. Last, but not least, GC–IRMS was used for measuring $\delta^{15}N$ values of N_2 and N_2O , separated from the same sample [151,152].

5. Hyphenated techniques

In recent years, the research efforts of different groups working in the field of GC–IRMS have focused on extending the scope of on-line CSIA towards the measurement of organic $^{18}O/^{16}O$ and organic $^2H/^1H$ isotope ratios. Consequently, research

was undertaken with the aim of high-precision measurement of two different elemental isotope ratios such as $^2\text{H}/^{18}\text{O}$, $^{13}\text{C}/^{18}\text{O}$ and $^{13}\text{C}/^{15}\text{N}$, from the same compound source in one analytical run.

By placing a PORAPLOT Q capillary column between the combustion reactor and the IRMS (which enabled us to separate N_2 from CO_2 by 100 s baseline to baseline), $^{15}\text{N}/^{14}\text{N}$ and $^{13}\text{C}/^{12}\text{C}$ isotope ratio from alanine, leucine and phenylalanine could be measured in one single analysis [153]. The excellent separation of N_2 from CO_2 provided ample time to switch IRMS ion source parameters from N_2 - to CO_2 -mode (Fig. 6).

In 1994, using a GC-based IRMS system, Brand et al. showed that CSIA of $^{18}\text{O}/^{16}\text{O}$ ratios was possible by converting oxygen-containing organic compounds on-line to CO by means of a pyrolytic reaction [154]. The on-line coupling of GC and IRMS via a pyrolysis interface (GC-Py-IRMS) was used for the simultaneous determination of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values for vanilla from different origins [155]. Farquhar et

al. converted bulk plant matter into N_2 and CO by an automated on-line pyrolysis-based reaction using nickelized carbon at about 1100°C and separating N_2 from CO post-pyrolysis in a GC fitted with a 5-Å molecular sieve PLOT column [156].

Independently, Begley and Scrimgeour reported on high-precision $\delta^2\text{H}$ and $\delta^{18}\text{O}$ measurement for water and VOCs by using 20% nickelized carbon to generate both H_2 and CO at temperatures of between 1050 and 1100°C [157]. Precisions were S.D. ($\delta^2\text{H}$) = 2‰ and S.D. ($\delta^{18}\text{O}$) = 0.3‰ for samples ranging from urine, water and VOCs. Their pyrolysis system was based on earlier work that was aimed at simultaneous $\delta^2\text{H}$ and $\delta^{18}\text{O}$ determination from small water and urine samples ($0.5\ \mu\text{l}$) [158]. Common to both studies was the use of a novel IRMS with the high dispersion necessary for separation of the $^2\text{H}^1\text{H}^+$ and $^4\text{He}^+$ ion beams. This novel high mass dispersion IRMS has been described in detail by Prosser and Scrimgeour [159]. Very recently, this high mass dispersion IRMS was coupled to a

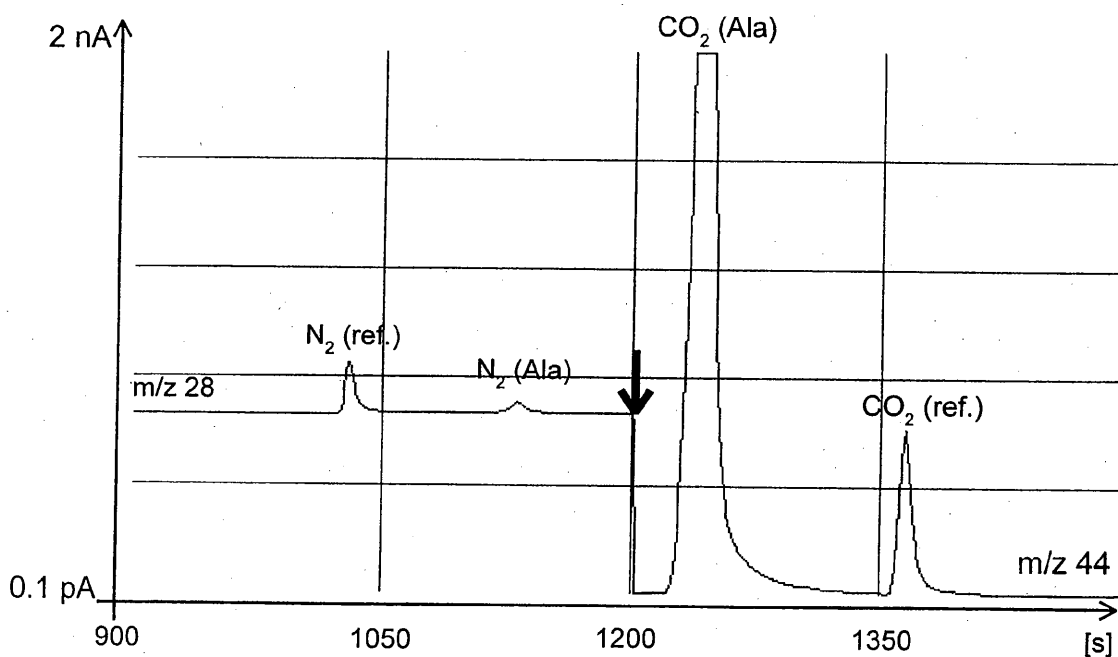


Fig. 6. Dual isotope measurement of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ from alanine (as *N*-acetyl, *O*-propyl derivative) during the same analysis. The arrow indicates when the ion source parameters were switched from-nitrogen mode ($m/z\ 28$) to carbon dioxide mode ($m/z\ 44$). A 100-s baseline to baseline separation of N_2 from CO_2 was achieved by passing the combustion products past-reduction through a PORAPLOT Q column, held at 35°C . The results obtained from dual-isotope analyses ($\delta^{15}\text{N}$, $-7.78 \pm 0.10\text{‰}$ vs air; $\delta^{13}\text{C}$, $-40.22 \pm 0.14\text{‰}$ vs PDB) were in good agreement with those obtained from separate analyses ($\delta^{15}\text{N}$, $-7.86 \pm 0.38\text{‰}$ vs air; $\delta^{13}\text{C}$, $-40.20 \pm 0.21\text{‰}$ vs PDB).

GC via a pyrolysis interface including a 5-Å molecular sieve PLOT column to achieve CSIA for ^2H of fatty acids. Preliminary $\delta^2\text{H}$ values for 16:0 and 18:1 fatty acids (as methyl esters) from tuna oil given in a Technical Brochure were -148.5 ± 4.1 and $-155.3 \pm 1.0\text{‰}$ (vs. VSMOV), respectively [160].

A different approach to CSIA for H of organic compounds such as ethyl benzene and cyclohexanone was published by Tobias and Brenna. Initially using a two-stage reactor interface (CuO at 850°C followed by Ni held at 950°C) [161] they found that better precision for $\delta^2\text{H}$ was achieved by employing an empty alumina tube held at about 1150°C [162]. Because their IRMS was not capable of fully resolving analyte $^2\text{H}^1\text{H}$ from excess ^4He carrier gas, they used a heated Pd filter in conjunction with a make-up pressure unit to prevent He from entering the IRMS while selectively admitting only hydrogen through the Pd foil membrane into the ion source [163].

The measurement of intramolecular variations in isotopic abundance due to kinetic isotope effects during biosynthesis is another recent development to extend the scope of GC-IRMS. The group around Schmidt employed ^{13}C isotope pattern analysis for distinction of natural compounds from corresponding synthetic products [53,164]. In 1997, an on-line pyrolysis system for position-specific isotope analysis (PSIA) of selected compounds from a complex mixture was described in detail by Corso and Brenna [165]. They coupled a GC (GC-1) for sample separation prior to pyrolysis to the GC (GC-2) separating pyrolytic products of the selected sample compound. Furthermore, they installed a valve into GC-2 to permit separated pyrolysis fragments to be admitted to an organic MS for structure analysis of these fragments.

6. Conclusions

Despite recent advances, many fundamental challenges for improved instrumentation still remain, most notably developments leading to (a) quantitative sample conversion to achieve high-precision CSIA for nitrogen; (b) routine CSIA of hydrogen isotopes after gas chromatographic separation; and (c) the routine application of PSIA to detect intramolecular isotope patterns. Continuing improve-

ments in accuracy, precision and abundance sensitivity of GC-IRMS, accompanied by increased user-friendliness, will ensure that this technique will cement its role as an important and unique tool of analytical mass spectrometry.

The sheer number of applications, as well as their wide spectrum clearly demonstrates that state-of-the-art GC-C-IRMS instruments are already powerful tools providing quantitative and qualitative information that cannot be obtained by other means.

Acknowledgements

The author gratefully acknowledges financial support through an industrial grant awarded by Europa Scientific Ltd (Crewe, UK). Many thanks are due to Professor Michael J. Rennie for advise and helpful discussions. The author is indebted to Dr Helen F Kemp for critical reading of the manuscript.

References

- [1] M. Sano, Y. Yotsui, H. Abe, S. Sasaki, *Biomed. Mass Spectrom.* 3 (1976) 1–3.
- [2] D.E. Matthews, J.M. Hayes, *Anal. Chem.* 50 (1978) 1465–1473.
- [3] A. Barrie, J. Bricout, J. Koziat, *Biomed. Mass Spectrom.* 11 (1984) 439–447.
- [4] J.R. Bacon, J.S. Crain, A.W. McMahon, J.G. Williams, *J. Anal. Atomic Spectrom.* 12 (1997) R407–R448.
- [5] M. Rautenschlein, K. Habfast, W. Brand, in: T.E. Chapman, R. Berger, D.J. Reijngoud, A. Okken (Eds.), *Stable Isotopes in Paediatric, Nutritional and Metabolic Research*, Intercept Ltd, Andover, UK, 1990, pp. 133–148.
- [6] D.A. Merritt, K.H. Freeman, M.P. Ricci, S.A. Studley, J.M. Hayes, *Anal. Chem.* 67 (1995) 2461–2473.
- [7] P.A. Eakin, A.E. Fallick, J. Gerc, *Chem. Geol.* 101 (1992) 71–79.
- [8] K.J. Leckrone, J.M. Hayes, *Anal. Chem.* 70 (1998) 2737–2744.
- [9] Y. Cherrah, J.B. Falconnet, M. Desage, J.L. Brazler, R. Zini, J.P. Tillement, *Biomed. Environ. Mass Spectrom.* 14 (1987) 653–657.
- [10] M. Matucha, W. Jokisch, P. Verner, G. Anders, *J. Chromatogr.* 588 (1991) 251–258.
- [11] M. Matucha, in: J. Allen (Ed.), *Synthesis and Applications of Isotopically Labelled Compounds*, Wiley, New York, 1995, pp. 489–494.

- [12] W. Meier-Augenstein, P.W. Watt, C.-D. Langhans, J. Chromatogr. A 752 (1996) 233–241.
- [13] K.J. Goodman, J.T. Brenna, Anal. Chem. 66 (1994) 1294–1301.
- [14] K.J. Goodman, J.T. Brenna, J. Chromatogr. A. 689 (1995) 63–68.
- [15] J.T. Brenna, T.N. Corso, H.J. Tobias, R.J. Caimi, Mass Spectrom. Rev. 16 (1997) 227–258.
- [16] K. Schumacher, H. Turgeon, A. Mosandl, Phytochem. Anal. 6 (1995) 258–261.
- [17] Y. Khalfallah, S. Normand, S. Tissot, C. Pachiaudi, M. Beylot, J.P. Riou, Biol. Mass Spectrom. 22 (1993) 707–711.
- [18] H. Demmelmair, H.-L. Schmidt, Isotopenpraxis Environ. Health Stud. 29 (1993) 237–250.
- [19] G. Rieley, Analyst 119 (1994) 915–919.
- [20] W. Meier-Augenstein, LC·GC 15 (1997) 244–253.
- [21] R.J. Caimi, L.H. Houghton, J.T. Brenna, Anal. Chem. 66 (1994) 2989–2991.
- [22] D.A. Merritt, W.A. Brand, J.M. Hayes, Org. Geochem. 21 (1994) 573–583.
- [23] R.J. Caimi, J.T. Brenna, J. Am. Soc. Mass Spectrom. 7 (1996) 605–610.
- [24] W. Meier-Augenstein, Rapid Commun. Mass Spectrom. 11 (1997) 1775–1780.
- [25] K. Grob, in: Making and Manipulating Capillary Columns for Gas Chromatography, Hüthig, Heidelberg, 1986, p. 193.
- [26] S. Nitz, B. Weinreich, F. Drawert, J. High Resolut. Chromatogr. 15 (1992) 387–391.
- [27] H. Casabianca, J.B. Graff, P. Jame, C. Perruchietti, M. Chastrette, J. High Resolut. Chromatogr. 18 (1995) 279–285.
- [28] D. Juchelka, T. Beck, H. Hener, F. Dettmar, A. Mosandl, J. High Resolut. Chromatogr. 21 (1998) 145–151.
- [29] S.E. Woodbury, R.P. Evershed, J.B. Rossel, J. Chromatogr. A 805 (1998) 249–257.
- [30] K.J. Goodman, Anal. Chem. 70 (1998) 833–837.
- [31] G. Rieley, J.W. Collister, B. Stern, G. Eglinton, Rapid Commun. Mass Spectrom. 7 (1993) 488–491.
- [32] J.W. Collister, G. Rieley, B. Stern, G. Eglinton, B. Fry, Org. Geochem. 21 (1994) 619–627.
- [33] Y.X. Liang, W.S. White, L.H. Yao, R.E. Serfass, J. Chromatogr. A 800 (1998) 51–58.
- [34] H.J. Lockheart, P.F. Van Bergen, R.P. Evershed, Org. Geochem. 26 (1997) 137–153.
- [35] A. Bernreuther, J. Koziat, P. Brunerie, G. Krammer, N. Christoph, P. Schreier, Z. Lebensm. Unters. Forsch. 191 (1990) 299–301.
- [36] A. Mosandl, U. Hener, H.-G. Schmarr, M. Rautenschlein, J. High Resolut. Chromatogr. 13 (1990) 528–531.
- [37] R. Braunsdorf, U. Hener, S. Stein, A. Mosandl, Z. Lebensm. Unters. Forsch. 197 (1993) 137–141.
- [38] A. Mosandl, Food. Rev. Int. 11 (1995) 597–664.
- [39] U. Hener, S. Faulhaber, P. Kreis, A. Mosandl, Pharmazie 50 (1995) 60–62.
- [40] C. Frank, A. Dietrich, U. Kremer, A. Mosandl, J. Agric. Food Chem. 43 (1995) 1634–1637.
- [41] D. Juchelka, A. Mosandl, Pharmazie 51 (1996) 417–422.
- [42] R. Braunsdorf, U. Hener, G. Przibilla, S. Piecha, A. Mosandl, Z. Lebensm. Unters. Forsch. 197 (1991) 24–28.
- [43] S. Faulhaber, U. Hener, A. Mosandl, J. Agric. Food Chem. 45 (1997) 2579–2583.
- [44] S. Faulhaber, U. Hener, A. Mosandl, J. Agric. Food Chem. 45 (1997) 4719–4725.
- [45] B. Faber, B. Krause, A. Mosandl, J. Essent. Oil Res. 7 (1995) 123–131.
- [46] V. Karl, A. Dietrich, A. Mosandl, Phytochem. Anal. 5 (1994) 32–37.
- [47] R. Braunsdorf, U. Hener, D. Lehmann, A. Mosandl, Dtsch. Lebensm. Rundsch. 87 (1991) 277–280.
- [48] A. Mosandl, R. Braunsdorf, G. Bruche, A. Dietrich, U. Hener, V. Karl, T. Köpke, P. Kreis, D. Lehmann, B. Maas, ACS Symp. Ser. 596 (1995) 94–112.
- [49] H.L. Schmidt, M. Butzenlechner, A. Rossmann, S. Schwarz, H. Kexel, K. Kempe, Z. Lebensm. Unters. Forsch. 196 (1993) 105–110.
- [50] M. Gensler, A. Rossmann, H.L. Schmidt, J. Agric. Food Chem. 43 (1995) 2662–2666.
- [51] D. Weber, M. Gensler, H.L. Schmidt, Isotopes Environ. Health Stud. 33 (1997) 151–155.
- [52] A. Rossmann, H.L. Schmidt, Anal. Chim. Acta 347 (1997) 359–368.
- [53] D. Weber, H. Kexel, H.L. Schmidt, J. Agric. Food Chem. 45 (1997) 2042–2046.
- [54] D. Weber, A. Rossmann, S. Schwarz, H.L. Schmidt, Z. Lebensm. Unters. Forsch. A 205 (1997) 158–164.
- [55] M.J. Dennis, R.C. Massey, T. Bigwood, Analyst 119 (1994) 2057–2060.
- [56] S.E. Woodbury, R.P. Evershed, J.B. Rossell, R.E. Griffith, P. Farnell, Anal. Chem. 67 (1995) 2685–2690.
- [57] S.E. Woodbury, R.P. Evershed, J.B. Rossell, J. Am. Oil Chem. Soc. 75 (1998) 371–379.
- [58] S. Kelly, I. Parker, M. Sharman, J. Dennis, I. Goodall, Food Chem. 59 (1997) 181–186.
- [59] F. Angerosa, L. Camera, S. Cumitini, G. Gleixner, F. Reniero, J. Agric. Food Chem. 45 (1997) 3044–3048.
- [60] M. Desage, R. Guilluy, J.L. Brazier, H. Chaudron, J. Girard, H. Cherpin, J. Jumeau, Anal. Chim. Acta 247 (1991) 249–254.
- [61] F. Mas, B. Beemsterboer, A.C. Veltkamp, A.M.A. Verweij, Forensic Sci. Int. 71 (1995) 225–231.
- [62] M. Becchi, R. Aguilera, Y. Farizon, M.M. Flament, H. Casabianca, P. James, Rapid Commun. Mass Spectrom. 8 (1994) 304–308.
- [63] R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic, H.G. Pope, J. Mass Spectrom. 31 (1996) 169–176.
- [64] C.H.L. Shackleton, A. Phillips, T. Chang, Y. Li, Steroids 62 (1997) 379–387.
- [65] C.H.L. Shackleton, E. Roitman, A. Phillips, T. Chang, Steroids 62 (1997) 665–673.
- [66] R. Aguilera, M. Becchi, L. Mateus, M.A. Popot, Y. Bonnaire, H. Casabianca, C.K. Hatton, J. Chromatogr. B 702 (1997) 85–91.
- [67] D.M. Jones, J.F. Carter, G. Eglinton, E.J. Jumeau, C.S. Fenwick, Biol. Mass Spectrom. 20 (1991) 641–646.

- [68] V. Hauke, R. Graff, P. Wehrung, J.M. Trendel, P. Albrecht, A. Riva, G. Hopfgartner, F.O. Gulacar, A. Buchs, P.A. Eakin, *Geochim. Cosmochim. Acta* 56 (1992) 3595–3602.
- [69] M.E.C. Moers, D.M. Jones, P.A. Eakin, A.E. Fallick, H. Griffiths, S.R. Larter, *Org. Biochem.* 20 (1993) 927–933.
- [70] R. Ishiwatari, M. Uzaki, K. Yamada, *Org. Geochem.* 21 (1994) 801–808.
- [71] A.J. Bakel, P.H. Ostrom, N.E. Ostrom, *Org. Geochem.* 21 (1994) 595–602.
- [72] M. Bjoroy, P.B. Hall, R.P. Moe, *Org. Geochem.* 22 (1994) 355–381.
- [73] Y.S. Huang, R. Bol, D.D. Harkness, P. Ineson, G. Eglinton, *Org. Geochem.* 24 (1996) 273–287.
- [74] K.J. Ficken, K.E. Barber, G. Eglinton, *Org. Geochem.* 28 (1998) 217–237.
- [75] M. Bjoroy, K. Hall, P. Gillyon, J. Jumeau, *Chem. Geol.* 93 (1991) 13–20.
- [76] A. Wilhelms, S.R. Larter, K. Hall, *Org. Geochem.* 21 (1994) 751–760.
- [77] E. Lichtfouse, H. Budzinski, P. Garrigues, T.I. Eglinton, *Org. Geochem.* 26 (1997) 353–359.
- [78] S.A. Macko, M.H. Engel, Y.R. Qian, *Chem. Geol.* 114 (1994) 365–379.
- [79] J.A. Silfer, Y. Qian, S.A. Macko, M.H. Engel, *Org. Geochem.* 21 (1994) 603–609.
- [80] M.H. Engel, S.A. Macko, Y. Qian, J.A. Silfer, *Adv. Space Res.* 15 (1994) 99–106.
- [81] J. Pulchan, T.A. Abrajano, R. Helleur, *J. Anal. Appl. Pyrol.* 42 (1997) 135–150.
- [82] K.H. Freeman, J.M. Hayes, J.M. Trendel, P. Albrecht, *Nature* 343 (1990) 254–256.
- [83] E. Lichtfouse, G. Berthier, S. Houot, E. Barriuso, V. Bergheaud, T. Vallaeys, *Org. Geochem.* 23 (1995) 849–852.
- [84] G.A. Logan, J.M. Hayes, G.B. Hieshima, R.E. Summons, *Nature* 376 (1995) 53.
- [85] R.P. Evershed, K.I. Arnot, J. Collister, G. Eglinton, S. Charters, *Analyst* 119 (1994) 909–914.
- [86] R.P. Evershed, A.W. Stott, A. Raven, S.N. Dudd, S. Charters, A. Leyden, *Tetrahedron Lett.* 36 (1995) 8875–8878.
- [87] A.M. Raven, P.F. Bergen, A.W. Stott, S.N. Dudd, R.P. Evershed, *J. Anal. Appl. Pyrol.* 40–41 (1997) 267–285.
- [88] R.P. Evershed, H.R. Mottram, S.N. Dudd, S. Charters, A.W. Stott, G.J. Lawrence, A.M. Gibson, A. Conner, P.W. Blinckhorn, V. Reeves, *Naturwissenschaften* 84 (1997) 402–406.
- [89] K. O'Donoghue, A. Clapham, R.P. Evershed, T.A. Brown, *Proc. Roy. Soc. London B* 263 (1996) 541–547.
- [90] A.W. Stott, R.P. Evershed, N. Tuross, *Org. Geochem.* 26 (1997) 99–103.
- [91] A.W. Stott, R.P. Evershed, *Anal. Chem.* 68 (1996) 4402–4408.
- [92] R.P. Evershed, S.J. Vaughan, S.N. Dudd, J.S. Soles, *Antiquity* 71 (1997) 979–985.
- [93] Y.Q. Zeng, H. Mukai, H. Bandow, Y. Nojiri, *Anal. Chim. Acta* 289 (1994) 195–204.
- [94] S.A. Bailis, K. Hall, E.J. Jumeau, *Org. Geochem.* 21 (1994) 777–785.
- [95] D.A. Merritt, J.M. Hayes, D.J. DesMarais, *J. Geophys. Res.* 100 (1995) 1317–1326.
- [96] M. Uzaki, K. Yamada, R. Ishiwatari, *Geochem. J.* 27 (1993) 385–389.
- [97] L. Mansuy, R.P. Philp, J. Allen, *Environ. Sci. Technol.* 31 (1997) 3417–3425.
- [98] B. Jovancicevic, L. Tasic, H. Wehner, E. Faber, N. Susic, P.P. Polic, *Fresenius Environ. Bull.* 6 (1997) 11–12.
- [99] L.M. Dowling, C.J. Boreham, J.M. Hope, A.P. Murray, R.E. Summons, *Org. Geochem.* 23 (1995) 729–737.
- [100] M. Whittaker, S.J.T. Pollard, T.E. Fallick, *Environ. Technol.* 16 (1995) 1009–1033.
- [101] M. Whittaker, S.J.T. Pollard, A.E. Fallick, T. Preston, *Environ. Pollut.* 94 (1996) 195–203.
- [102] C. Mcrae, G.D. Love, I.P. Murray, C.E. Snape, A.E. Fallick, *Anal. Commun.* 33 (1996) 331–333.
- [103] V.P. O'Malley, A. Starck, T.A. Abrajano, J. Hellou, L. Winsor, *Polycycl. Aromat. Comp.* 9 (1996) 93–100.
- [104] D.C. Ballentine, S.A. Macko, V.C. Turekian, W.P. Gilhooly, B. Martincigh, *Org. Geochem.* 25 (1996) 97–104.
- [105] M.I. Bird, R.E. Summons, M.K. Gagan, Z. Roksandic, L. Dowling, J. Head, L.K. Fifield, R.G. Cresswell, D.P. Johnson, *Geochim. Cosmochim. Acta* 59 (1995) 2853–2857.
- [106] H. Naraoka, K. Yamada, R. Ishiwatari, *Geochem. J.* 29 (1995) 189–195.
- [107] H. Demmelmair, U. Vonschenck, E. Behendt, T. Sauerwald, B. Koletzko, *J. Paediatr. Gastroenterol. Nutr.* 21 (1995) 31–36.
- [108] V.P. Carnielli, D.J.L. Wattimena, I.H.T. Luijendijk, A. Boerlage, H.J. Degnhart, P.J.J. Sauer, *Paediatr. Res.* 40 (1996) 169–174.
- [109] V.P. Carnielli, E.J. Sulkers, C. Moretti, J.L.D. Wattimena, J.B. Vangoudoever, H.J. Degnhart, F. Zaccchello, P.J.J. Sauer, *Metabolism* 43 (1994) 1287–1292.
- [110] J. Koziat, P. Gross, G. Debry, M.J. Royer, *Biol. Mass Spectrom.* 20 (1991) 777–782.
- [111] C.C. Metges, K. Kempe, G. Wolfram, *Biol. Mass Spectrom.* 23 (1994) 295–301.
- [112] C. Binnert, M. Laville, C. Pachiaudi, V. Rigalleau, M. Beylot, *Lipids* 30 (1995) 869–873.
- [113] M. Stolinski, J.L. Murphy, A.E. Jones, A.A. Jackson, S.A. Wootton, *Lipids* 32 (1997) 337–340.
- [114] Z.K. Guo, S. Nielsen, B. Burguera, M.D. Jensen, *J. Lipid Res.* 38 (1997) 1888–1895.
- [115] Z.K. Guo, M.D. Jensen, *J. Appl. Physiol.* 84 (1998) 1674–1679.
- [116] N. Brossard, C. Pachiaudi, M. Croset, S. Normand, J. Lecerc, V. Chirouze, J.P. Riou, J.L. Tayot, M. Lagarde, *Anal. Biochem.* 220 (1994) 192–199.
- [117] R.C. Sheaff, H.M. Su, L.A. Keswick, J.T. Brenna, *J. Lipid Res.* 36 (1995) 998–1008.
- [118] M. Croset, N. Brossard, C. Pachiaudi, S. Normand, J. Lecerc, V. Chirouze, J.P. Riou, J.L. Tayot, M. Lagarde, *Lipids* 31 (1996) S109–115.
- [119] H. Demmelmair, T. Sauerwald, B. Koletzko, T. Richter, *Eur. J. Paediatr.* 156 (1997) S70–74.
- [120] N. Brossard, M. Croset, S. Normand, J. Pousin, J. Lecerc, M. Laville, J.L. Tayot, M. Lagarde, *J. Lipid Res.* 38 (1997) 1571–1582.

- [121] S.C. Cunnane, G. Moine, S.S. Likhodii, J. Vogt, T.N. Corso, J.T. Brenna, H. Demmelmair, B. Koletzko, K.H. Tovar, G. Kohn, G. Sawatzki, R. Muggli, *Lipids* 32 (1997) 211–217.
- [122] S. Tissot, S. Normand, R. Guilluy, C. Pachiaud, M. Beylot, M. Laville, R. Cohen, R. Mornex, J.P. Rieu, *Diabetologica* 33 (1990) 449–456.
- [123] B.J. Jandrain, N. Pallikarakis, S. Normand, F. Pimay, M. Lacroix, F. Mosora, C. Pachiaud, J.F. Gautier, A.J. Scheen, J.P. Riou, P.J. Lebeuvre, *J. Appl. Physiol.* 74 (1993) 2146–2154.
- [124] C. Rambal, C. Pachiaud, S. Normand, J.P. Riou, P. Louisot, A. Martin, *Carbohydr. Res.* 236 (1992) 29–37.
- [125] C. Rambal, C. Pachiaud, S. Normand, J.P. Riou, P. Louisot, A. Martin, *Ann. Nutr. Metab.* 39 (1995) 143–151.
- [126] F. Pont, L. Duviard, C. Maugeais, A. Athias, L. Persegol, P. Gambert, B. Verges, *Anal. Biochem.* 248 (1997) 277–287.
- [127] J.E.H. Bunt, L.J. Zimmermann, J.L.D. Wattimena, R.H. VanBeek, P.J. Sauer, V.P. Carnielli, *Am. J. Resp. Crit. Care Med.* 157 (1998) 810–814.
- [128] M. Beylot, F. David, Y. Khalfallah, S. Normand, V. Large, H. Brunengraber, *Biol. Mass Spectrom.* 23 (1994) 510–513.
- [129] W.D. Kloppenburg, B.G. Wolthers, F. Stellaard, H. Elzinga, T. Tepper, P.E. DeJong, R.M. Huisman, *Clin. Sci.* 93 (1997) 73–80.
- [130] W. Meier-Augenstein, D. Rating, G.F. Hoffmann, U. Wendel, U. Matthiesen, O. Schadewaldt, *Isotopes Environ. Health Stud.* 31 (1995) 261–266.
- [131] D.J. Reijngoud, G. Hellstern, H. Elzinga, M.G. De Sain-Vander, A. Okken, F. Stellaard, *J. Mass Spectrom.* 33 (1998) 621–626.
- [132] W.H. Hartl, H. Demmelmair, K.W. Jauch, H.L. Schmidt, B. Koletzko, F.W. Schildberg, *Am. J. Physiol. Endocrinol. Metab.* 35 (1997) E796–802.
- [133] W.H. Hartl, H. Demmelmair, K.W. Jauch, B. Koletzko, F.W. Schildberg, *Ann. Surg.* 227 (1998) 390–397.
- [134] R. Roscher, A. Hilkert, M. Gessner, E. Schindler, P. Schreier, W. Schwab, *Z. Lebensm. Unters. Forsch. A* 204 (1997) 198–201.
- [135] J.T. Brenna, T.N. Corso, H.J. Tobias, R.J. Caimi, *Mass Spectrom. Rev.* 16 (1997) 227–258.
- [136] K.J. Goodman, J.T. Brenna, *Anal. Chem.* 64 (1992) 1088–1095.
- [137] Z.K. Guo, A.H. Luke, W.P. Lee, D. Schoeller, *Anal. Chem.* 65 (1993) 1954–1959.
- [138] S.K. Rhee, H.M. Su, L.A. Keswick, J.T. Brenna, *Am. J. Clin. Nutr.* 65 (1997) 451–458.
- [139] C. Menand, E. Pouteau, S. Marchini, P. Mauge, M. Krempf, D. Darmaun, *J. Mass Spectrom.* 32 (1997) 1094–1099.
- [140] G. Dube, A. Henrion, W. Richter, *Metrologia* 34 (1997) 83–86.
- [141] G. Dube, A. Henrion, R. Ohlendorf, W. Richter, *Rapid Commun. Mass Spectrom.* 12 (1998) 28–32.
- [142] T. Preston, C. Slater, *Proc. Nutr. Soc.* 53 (1994) 363–372.
- [143] D.A. Merritt, J.M. Hayes, *J. Am. Soc. Mass Spectrom.* 5 (1994) 387–397.
- [144] D. Hoffmann, K. Jung, H.J. Segschneider, M. Gehre, G. Schuurmann, *Isotopes Environ. Health Stud.* 31 (1995) 267–375.
- [145] H.J. Segschneider, D. Hoffmann, G. Schmidt, R. Russon, *Isotopes Environ. Health Stud.* 31 (1995) 315–325.
- [146] C.C. Metges, K.J. Petzke, U. Henning, *J. Mass Spectrom.* 31 (1996) 367–376.
- [147] T. Preston, K.C.H. Fearon, D.C. McMillan, F.P. Winstanley, C. Slater, A. Shenkin, D.C. Carter, *Br. J. Surg.* 82 (1995) 229–234.
- [148] C.L. Williams, T. Preston, M. Hossack, C. Slater, K.E.L. McColl, *FEMS Immunol. Med. Microb.* 13 (1996) 87–94.
- [149] I.A. Simpson, R. Bol, S.J. Dockrill, K.J. Peteke, R.P. Evershed, *Archaeol. Prospect.* 4 (1997) 147–152.
- [150] C.C. Metges, K.J. Petzke, *Anal. Biochem.* 247 (1997) 158–164.
- [151] P.D. Brooks, G.J. Atkins, *Isotopenpraxis* 28 (1992) 106–109.
- [152] A. Barrie, S.T. Brookes, S.J. Prosser, S. Debney, *Fertil. Res.* 42 (1995) 43–59.
- [153] M.J. Rennie, W. Meier-Augenstein, P.W. Watt, A. Patel, I.S. Begley, C.M. Scrimgeour, *Biochem. Soc. Trans.* 24 (1996) 927–932.
- [154] W.A. Brand, A.R. Tegtmeier, A. Hilkert, *Org. Geochem.* 21 (1994) 585–594.
- [155] U. Hener, W.A. Brand, A.W. Hilkert, D. Juchelka, A. Mosandl, F. Pödebrand, *Z. Lebensm. Unters. Forsch. A* 206 (1998) 230–232.
- [156] G.D. Farquhar, B.K. Henry, J.M. Styles, *Rapid Commun. Mass Spectrom.* 11 (1997) 1554–1560.
- [157] I. Begley, C. Scrimgeour, *Anal. Chem.* 69 (1997) 1530–1535.
- [158] I. Begley, C. Scrimgeour, *Rapid Commun. Mass Spectrom.* 10 (1996) 969–973.
- [159] S.J. Prosser, C. Scrimgeour, *Anal. Chem.* 67 (1995) 1992–1997.
- [160] Hydra-GC, Technical Brochure H/7/98, Crewe, Europa Scientific, UK.
- [161] H.J. Tobias, J.T. Brenna, *Anal. Chem.* 68 (1996) 3002–3007.
- [162] H.J. Tobias, J.T. Brenna, *Anal. Chem.* 69 (1997) 3148–3152.
- [163] H.J. Tobias, K.J. Goodman, C.E. Blacken, J.T. Brenna, *Anal. Chem.* 67 (1995) 2486–2492.
- [164] T. Weilacher, G. Gleixner, H.L. Schmidt, *Phytochemistry* 41 (1996) 1073–1077.
- [165] T.N. Corso, J.T. Brenna, *Proc. Natl. Acad. Sci. USA* 94 (1997) 1049–1053.
- [166] A.R. Newman, *Anal. Chem.* 68 (1996) 373A–377A.

Routine Analysis by High Precision Gas Chromatography/Mass Selective Detector/Isotope Ratio Mass Spectrometry to 0.1 Parts Per Mil

James A. Hall*, Johannes A. C. Barth and Robert M. Kalin

Environmental Engineering Research Centre, Department of Civil Engineering, Queens University Belfast, Belfast BT7 1NN, N. Ireland

Stable isotope methods are potentially quite useful for validating natural or enhanced mineral degradation of contaminants. For this reason, a continuous flow gas chromatograph (GC), isotope ratio mass spectrometer (IRMS) has been coupled with a quadrupole mass selective detector (MSD) to allow simultaneous mass spectral and stable carbon isotope ratio data to be obtained from a single chromatographic analysis. This allows the target contaminant and any extra-cellular degradation intermediates to be both qualified and quantified. Previously acceptable limits of precision (0.3 parts per mil) are undesirable given the small fractionation observed during aerobic degradation. To further understand the fate of organic contaminants and to gain information about the metabolic degradative pathway employed by a microorganism, routine isotopic analyses on a range of analytes have been performed. Quantities of sample producing mass-44 ion beam signal (I^{44}) of 2×10^{-10} to 1×10^{-8} A were analysed. When the IRMS was tuned for high sensitivity, ion source nonlinearities were overcome by peak height correction from an algorithm that was produced using known isotopic standards of varying concentrations. This led to sample accuracy of $<0.01\%$ and sample precision of 0.1% . Copyright © 1999 John Wiley & Sons, Ltd.

Received 26 January 1999; Revised 15 March 1999; Accepted 17 March 1999

The ability to elucidate small differences in the stable carbon isotopic composition of organic compounds by means of isotope ratio mass spectrometry¹ provides a powerful tool in studying processes that may discriminate one stable isotope over another. Biological isotope fractionation² opens a range of potential applications for this technique. This means that isotope effects can be used as a powerful tool for gaining information about chemical reactions and enzyme-catalysed degradation steps.^{3–5} A major development in the field was the coupling of a gas chromatograph (GC) to an IRMS⁶ by the introduction of a combustion interface (C). The principle difference between a standard IRMS and a GC/IRMS was the continuous on-line nature of the latter. Organic samples of interest can now be isolated and oxidised on-line by passing them through a copper oxide combustion tube. The combustion products are then purified to leave only target CO_2 derived from the sample carbon. This is fed directly into the IRMS, allowing separation and rapid determination of isotopic ratios for a range of organic compounds in complex matrices. However, this innovation initially compromised the precision of the instrument. Instrumental differences from dual inlet mass spectrometers meant that precision of the initial GC/IRMS and later described instruments⁷ of 0.5% was lower than the

$<0.1\%$ reported for the dual inlet instruments.² Reasons for the decrease in precision^{7,8} included:

- the difference in ion beam size between the reference peak and the sample peak due to the capability of the GC/IRMS to analyse a range of analytes from one sample,
- the quality and size of the combustion tube packing, and
- the performance and design of the cryogenic water trap.

It is therefore obvious that a better understanding of possible instrumental effects will be advantageous to any analytical system⁹ especially when it has the complexities of two or more instruments coupled together.

An IRMS has applications in a large area of environmental monitoring.^{10,11} However, the complexity of bio-remediation systems presents a large variety of possible compounds present in the sub-surface as target contaminants¹² and/or secondary metabolic breakdown products. It would therefore be beneficial to have an analytical system that provides structural information as well as accurate, precise carbon isotopic information about a target analyte from a single sample. Meier-Augenstein *et al.*¹³ described an instrument capable of performing this dual analysis by coupling an ion trap detector to a GC/C/IRMS. Our aim was to couple an existing GC/mass selective detector (MSD) system to an IRMS detector thus incorporating a quadrupole detector rather than an ion trap detector as described previously.¹³ Furthermore, it is desirable to achieve accurate and precise results over a large range of sample sizes.

*Correspondence to: J. A. Hall, Environmental Engineering Research Centre, Department of Civil Engineering, Queens University Belfast, Belfast BT7 1NN, N. Ireland.

Contract/grant sponsor: QUESTOR Industrial Board.

Contract/grant sponsor: Queen's University Belfast, N. Ireland.

Contract/grant sponsor: EPSRC; Contract/grant number: L85183 and M26374.

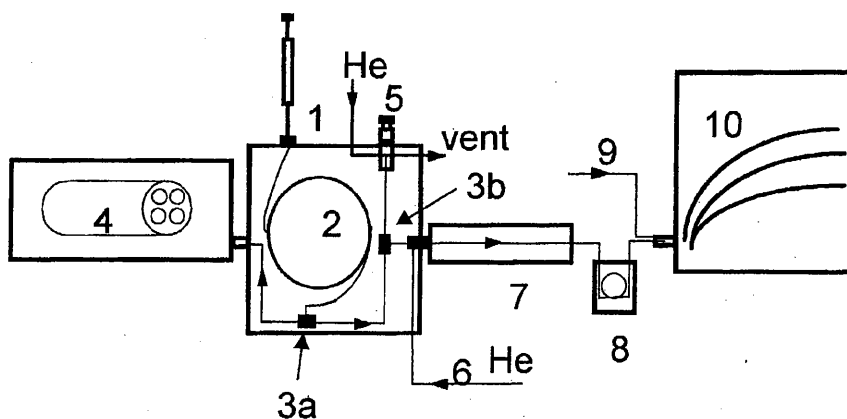


Figure 1. Instrument setup: 1 Injection port, 2 Gas chromatographic column, 3 a, b Split valves, 4 MSD with quadrupole filter, 5 Heart split valve, 6 Sample line helium, 7 combustion tube, 8 water trap, 9 Reference gas, 10 IRMS.

Metabolic intermediate products from degradation pathways can be formed in small amounts¹⁴ or be present for a short period of time.^{15,16} Sample considerations, coupled with the problem that some of the original chromatographic sample is diverted to the MSD, necessitates analysis of small samples producing mass 44 ion beam signals (I^{44}) between 1×10^{-10} and 1×10^{-8} A. At the time of writing the manufacturer's recommendation is that, after optimisation, analysis is only non-signal dependent over one order of magnitude (1×10^{-9} to 1×10^{-8} A).

In this paper we describe a GC/IRMS system coupled with a mass selective detector (MSD) that provides routine quantitative and qualitative analysis from the MSD and rapid carbon isotopic analysis using the IRMS (Fig. 1). We also outline a series of analyses on a variety of compounds and the development of a simple correction to allow routine analysis of trace compounds ($I^{44} = 2 \times 10^{-10}$ – 1×10^{-8} A) to an accuracy of 0.01‰ and to a precision of 0.1‰.

EXPERIMENTAL

Instrumentation

Figure 1 outlines the GC/MSD/IRMS system currently operating in the Environmental Research Centre (EERC) at Queen's University Belfast. The chromatographic unit is a CE 8000 Top gas chromatograph fitted with a Cryo 820 sub-ambient oven temperature unit (Thermoquest, Milan, Italy). The column effluent is then split inside the oven by a SGE GVF16-(2)004 ferrule (Fig. 1, item 3a). One output from this ferrule flows into the MD800 mass selective detector (Fig. 1, item 4) via a measured length of fused capillary tubing. The second effluent flow is connected to a second SGE GCF16(2)004 ferrule (Fig. 1, item 3b). This then splits the flow into two further capillaries. One of the capillaries flows into the heart split valve (Fig. 1, item 5) and the other into the combustion interface (Fig. 1, item 7). The sample is oxidised by fine CuO granules (0.61 mm) and passed through a cryogenic water trap (Fig. 1, item 8). This removes water from the oxidation products allowing purified CO₂ to flow into a bench-top VG Isoprime® IRMS (Micromass UK Ltd., Manchester, UK) (Fig. 1, item 10).

Standard preparation

Four compounds, decane, undecane, dodecane and methyl decanoate, (VG mix, obtained from Micromass UK Ltd.) were dissolved in hexane (15 ng/mL). This is used as an internal laboratory standard and routinely analysed as a quality assurance/control (QA/QC) to check the system.

Isotopically known Carrara marble¹⁷ derived CO₂ standards were made by dissolving a powdered aliquot of Carrara marble in excess phosphoric acid (95%). The acidification for the liberation of CO₂ was carried out in pre-evacuated, gas-tight vials. The vials were then kept at 25 °C for at least 4 hours to ensure CO₂ evolution under equilibrium conditions.

The phenol standards were obtained by dissolution of pure compound in ethyl acetate (0.47 mg/mL). The $\delta^{13}\text{C}$ value of phenol was also measured on a Prism III dual inlet IRMS. Ethyl acetate was used as the solvent to compare with the phenol extraction method used to study phenol utilisation and isotopic fractionation.¹⁸

Chromatographic conditions

The VG mix and phenol samples were injected with varying split ratios (30:1–166:1) and sample sizes (1–10 µL) to give the desired I^{44} intensity. Investigations have shown that split injection causes no isotopic fractionation.⁹ The CO₂ samples were injected in split mode using a gas-tight syringe. Helium (3 mL/min) was used as the carrier gas. The GC columns used were a DB5® 15 m × 0.22 mm (J & W Scientific, Folsom, CA, USA) for organic samples and a CP-Molsieve 5 Å 25 m × 0.32 mm (Chrompack, Middelburg, The Netherlands) for CO₂ samples.

RESULTS

Instrumental conditions

The identification of an analyte by its mass spectrum and the carbon isotopic ratio obtained from the same compound are unambiguously linked, because both sets of information are obtained from one injection. Figure 2 suggests that the column effluent is split effectively between the two detectors. The nonlinear trend between MSD and IRMS peak areas is thought to be an artefact of the MSD response

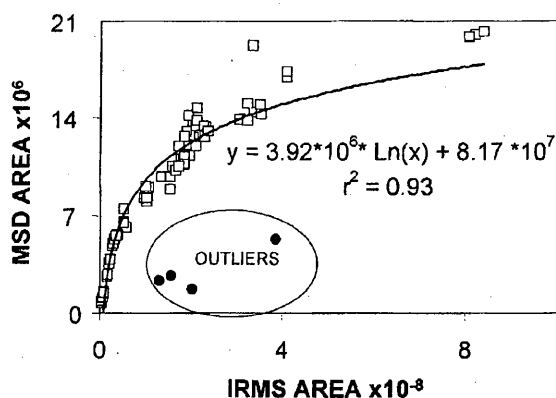


Figure 2. Trend of the peak areas of the IRMS and MSD.

Table 1. Measured $\delta^{13}\text{C}$ values of analytes at constant ^{44}I beam ($2\mu\text{L}$ injection, split 20:1) in comparison to their theoretical values including retention times (R_t) on the MSD and IRMS

| Component | Decane | Undecane | Dodecane | Methyl decanoate |
|-------------------|--------|----------|----------|------------------|
| | -28.34 | -26.61 | -28.50 | -30.42 |
| | -28.56 | -26.78 | -28.65 | -30.72 |
| | -28.54 | -26.71 | -28.57 | -30.50 |
| | -28.43 | -26.56 | -28.56 | -30.56 |
| | -28.57 | -26.72 | -28.59 | -30.51 |
| Average | -28.49 | -26.68 | -28.57 | -30.54 |
| 1σ | 0.10 | 0.09 | 0.05 | 0.11 |
| Theoretical | -28.61 | -26.70 | -28.63 | -30.47 |
| Difference | 0.12 | 0.02 | 0.06 | -0.07 |
| MSD R_t | 300.6 | 387.6 | 469.8 | 564.6 |
| $1\sigma^\dagger$ | 1.2 | 0.6 | 0.6 | 0.6 |
| IRMS R_t | 333.5 | 420 | 501.6 | 596.6 |
| 1σ | 2.1 | 2.5 | 2.8 | 2.7 |

$^\dagger 1\sigma$ - one standard deviation.

to increasing sample size. The MSD response is not linear above 1.4×10^7 total ion count (TIC) and this therefore creates the nonlinear effect at higher sample concentrations seen in Fig. 2.

Although the column flow is split and forced to flow through an excess length of capillary ($40\text{ cm} \times 75\text{ }\mu\text{m}$), the chromatographic resolution is not degraded. Likewise, despite being split twice by union valves, the resolution of the spectrum from the IRMS is not impaired, nor the integrity of the isotopic data harmed (Table 1). The main difficulty in using an MSD as a second detector with a GC-IRMS is obtaining the correct pressure balance.

With a normal GC-IRMS system, after solvent peak elution, the heart split valve closes, the sample line helium builds up a back pressure and forces the column effluent to flow with this excess helium along the combustion interface to the IRMS detector. However, we have added an extra capillary from the MSD that exerts a negative pressure on the initial split at the end of the column. This subsequently creates a delicate balance between three exerting pressures: (1) the negative pressure created by the MSD, (2) the pressure flow of column effluent, and (3) the pressure flow of sample line helium.

The balance of these pressures can be calculated using Eqn. 1 or empirically. A major problem to be noted was the

difficulty in placing the two capillaries in the union on the opposite side of the column fitting to achieve a balanced flow to both the MSD and the second split. Once this was achieved, the ideal column flow was elucidated ($2.0\text{--}3.5\text{ mL/min}$) to maintain chromatographic integrity and speed of analysis. The length of capillary from the first split to the MSD interface had to be of the correct diameter to maintain laminar flow. The relationship is outlined in the following modified Poiseuille's equation:

$$V_0 = 50747 \cdot \frac{d^4}{\eta l} \cdot \frac{\{(p_i + 14.7)^2 - (p_o + 14.7)^2\}}{(p_o + 14.7)} \cdot \frac{p_o}{p_m} \cdot \frac{(t_m + 273)}{(t_c + 273)} \quad (1)$$

where V_0 is the carrier gas volume (mL/min), η is the dynamic viscosity of the carrier gas (micropoises), l is the length of the capillary column (m), p_i is the carrier gas inlet pressure above atmospheric (psi), p_o is the carrier gas outlet pressure (psi), p_m is the pressure at which the measurement is made, t_m is the temperature at which the measurement can be made ($^\circ\text{C}$) and t_c is the temperature in the column. The important factor to note from Eqn. 1 is that the relationship between flow through the capillary and the diameter is proportional to d^4 . This means that for a small decrease in the diameter of the capillary to the MSD we observe a substantial decrease in the effective vacuum exerted on the split union.

Organic samples

Initially the VG mix was used to calibrate the instrument. The IRMS I^{44} sensitivity is greatly variable depending on variations in the set of source tuning parameters. This, coupled with the fact that the flow is split at the injection port, first union, and at the open split at the end of the combustion tube, suggests that the absolute mass of carbon entering the IRMS source is difficult to quantify. The sample is therefore described as nmoles on-column. A common term encountered in the literature describing the performance of an IRMS instrument is the 'linearity'.¹⁹ It is the authors' opinion that this term is vague and incorrectly used. The term 'linear' should describe the fact that the ion beam intensity is related by a linear (1st order polynomial) variation to the $\delta^{13}\text{C}$ value produced by that signal. IRMS manufacturers and operators generally use the term 'linear' to mean that the measured $\delta^{13}\text{C}$ value produced by the IRMS is independent of I^{44} intensity. A more correct term for this is signal independence and this is used herein. Table 1 shows that a $2\mu\text{L}$ injection at a split of 20:1 of VG mix (1.5 ng on-column) is required for an I^{44} of $6.5 \times 10^{-9}\text{ A}$. This is well within the manufacturer's specified signal independent range. The carbon isotopic ratios of the four compounds showed an average precision of 0.09% (1σ) and an accuracy error of $<0.03\%$. Table 1 also shows that the retention times on both detectors were very reproducible.

As stated earlier, our analytical system consists of a GC coupled to two detectors. It is therefore of prime importance to optimise the sensitivity of the IRMS to counter the effect of the extra split of column effluent to the MSD (Fig. 1, item 3a). This was achieved by setting the source tuning parameters to values that assured a high sensitivity response. The tuning parameters manipulated were the extraction voltage, half-plate differential voltage, z focus, ion repeller and electron volt setting.

Results outlined below demonstrate that the instrument

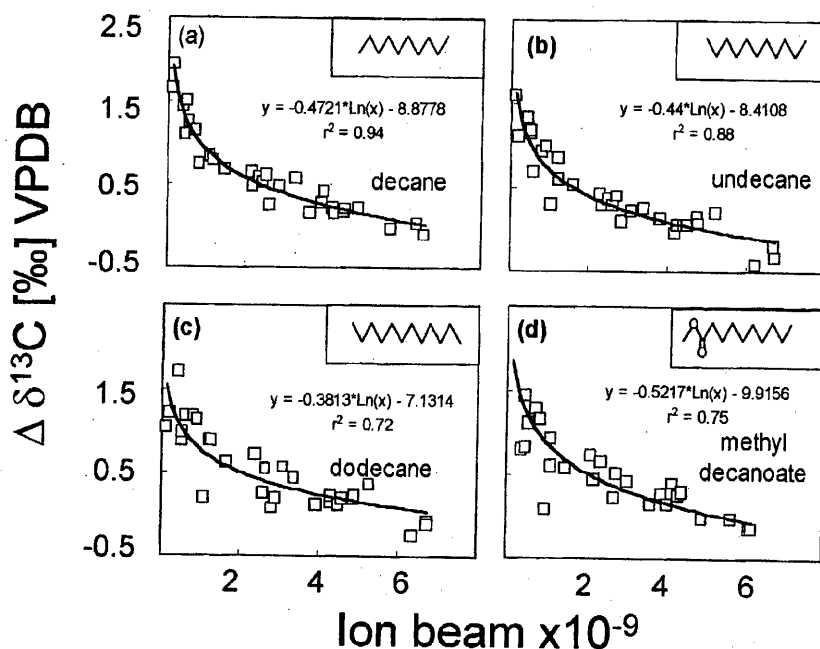


Figure 3. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta \delta^{13}\text{C}_{\text{DIC}}$) for decane, undecane, dodecane and methyl decanoate. The upper right corner of each graph shows the structure of the analyte.

produced $\delta^{13}\text{C}$ values that were signal dependent. Our initial investigations showed that although the $\delta^{13}\text{C}$ values produced for the VG mix were approximately non-signal dependent to $\pm 0.3\text{‰}$ for the ion beam intensities between 1×10^{-9} and 1×10^{-8} A, the sample accuracy and precision soon degraded significantly between 1×10^{-10} and 1×10^{-9} A. A series of injections was undertaken to produce I^{44} intensities of between 1×10^{-10} and 8×10^{-8} A. Figure 3 illustrates that the $\delta^{13}\text{C}$ values produced for decane, undecane, dodecane and methyl decanoate are exponentially dependent on the I^{44} intensity. The delta value (Δ) plotted represents the difference between the theoretical value given by Micromass UK Ltd. and the values recorded by the IRMS. All four compounds showed a positive Δ of between 1.6 and 2‰ when the ion beam signal was smaller than 5×10^{-10} A. This enrichment decreased with increasing I^{44} intensity. Figure 3 demonstrates that the theoretical $\delta^{13}\text{C}$ value is approached when the I^{44} intensity is between 5.5×10^{-9} and 7×10^{-9} A. This is because the $\delta^{13}\text{C}$ value of the reference gas used in the IRMS was cross-calibrated using VG mix at I^{44} intensities of 6×10^{-9} to 6.5×10^{-9} A. The relative molecular masses of these compounds varied from 142 to 172 Da. The logarithmic regression observed in Fig. 3 does not appear to be mass dependent. The MSD successfully identified all compounds plotted in Fig. 3 to at least a 93% confidence level.

Bioremediation of phenol is currently ongoing within the EERC.¹⁸ Phenol was therefore selected to study the effect of signal intensity on the $\delta^{13}\text{C}$ value recorded for a smaller molecule. Sample sizes analysed were again varied to produce I^{44} intensities of 1×10^{-10} – 1×10^{-8} A. Figure 4(a) illustrates that the $\delta^{13}\text{C}$ values produced were again

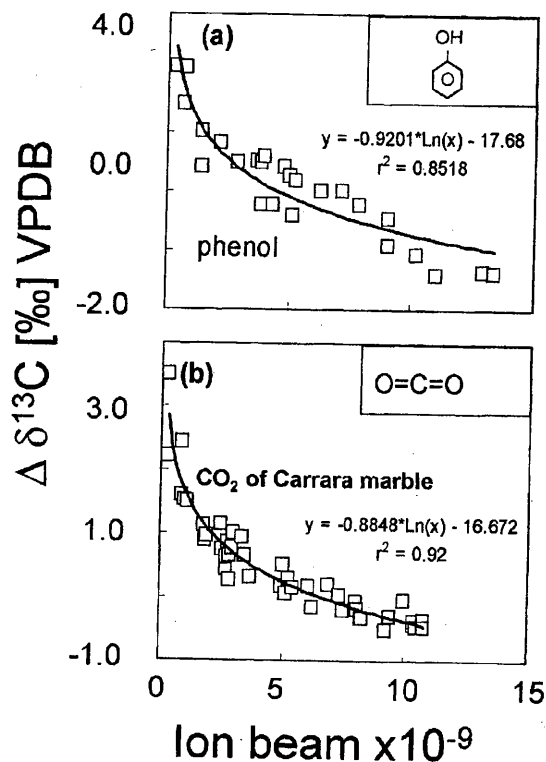


Figure 4. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta \delta^{13}\text{C}_{\text{DIC}}$) for phenol and CO_2 from Carrara marble. The upper right corner of each graph shows the structure of the analyte.

signal dependent. The $\delta^{13}\text{C}$ value becomes more enriched with decreasing sample size, reaching a maximum enrichment of 1.2‰ at an I^{44} intensity of 7×10^{-10} A. The theoretical $\delta^{13}\text{C}$ value of phenol (−28.5‰) is approached when the I^{44} intensity is 6.8×10^{-9} A.

CO₂ standards

Our aim was also to investigate the role of the combustion tube in the signal dependence trend observed. A series of CO₂ analyses derived from the acidification of Carrara marble were performed. Injecting CO₂ directly onto the column with identical operational conditions for the combustion interface obviously negates the necessity of CuO in the combustion interface to oxidise the carbon present in the sample. Figure 4(b) shows the $\delta^{13}\text{C}$ value plotted against ion beam size. The Carrara marble standard shows a maximum enrichment of 2.5‰ for an ion beam size of 3×10^{-10} A. The true value of the standard (2.4‰) is approached for an ion beam size of 6.5×10^{-9} A.

DISCUSSION

This investigation outlines the successful coupling of two independent detectors, an MSD and an IRMS to a GC. When using standard gas chromatography/mass spectrometric analysis it is important to optimise the detector to enable quantification and qualification of the smallest possible sample size. The IRMS used in our system has been optimised to operate at a high sensitivity. This now enables the quantification, qualification and $\delta^{13}\text{C}$ values of compounds to be recorded from one sample analysis.

After compromising the signal independence by tuning the IRMS for higher sensitivity, a logarithmic relationship occurs between the $\delta^{13}\text{C}$ value and the I^{44} intensity for all four compounds in Fig. 3. By manipulating the equation best describing the points ($y = m \cdot \ln(x) + c$) in Fig. 3, Eqn. 2 is derived:

$$\delta^{13}\text{C}_{\text{true}} = \delta^{13}\text{C}_{\text{actual}} - ((m \cdot \ln(I^{44})) + c) \quad (2)$$

We were then able to correct the $\delta^{13}\text{C}$ values obtained for any I^{44} between 2×10^{-10} and 7×10^{-9} A. Using this correction equation, the compounds in Fig. 3 produced $\delta^{13}\text{C}$ values to an accuracy of <0.01‰ and a precision of between 0.13 and 0.23‰. Corrected phenol $\delta^{13}\text{C}$ values were also calculated using Eqn. 2. Over two orders of magnitude the accuracy using this technique is <0.01‰ with a precision of 0.3‰. This precision is not particularly good. This could be an artefact of sample degradation over time. The results were obtained over two days and phenol has been shown to degrade as a function of time in ethyl acetate.²⁰

The $\delta^{13}\text{C}$ values of the organic compounds investigated all display a signal dependence relative to I^{44} intensity. A possible source of instrumental error could be the combustion interface.⁸ To elucidate whether this may be the case CO₂ was injected directly onto the column. The Carrara marble data showed the same logarithmic signal dependence shown by compounds that were injected on-column as liquids and combusted on the CuO interface. The $\delta^{13}\text{C}$ values could therefore also be corrected by Eqn. 2. When the correction was performed, the $\delta^{13}\text{C}$ values obtained were accurate to 0.01‰ with a precision of 0.25‰ from 2.2×10^{-10} to 1.1×10^{-8} A. The IRMS does have the potential for very

Table 2. Measured and corrected $\delta^{13}\text{C}$ values of decane at variable I^{44} beam intensities. The average of the latter shows a significant increase in precision and coincides with the theoretical value (high accuracy)

| I^{44} beam | $\delta^{13}\text{C}$ measured | $\delta^{13}\text{C}$ corrected |
|---------------|--------------------------------|---------------------------------|
| 1.19E-10 | −26.96 | −28.87 |
| 1.61E-10 | −26.68 | −28.45 |
| 4.00E-10 | −27.18 | −28.52 |
| 4.76E-10 | −27.51 | −28.77 |
| 4.82E-10 | −27.11 | −28.36 |
| 5.49E-10 | −27.36 | −28.55 |
| 7.47E-10 | −27.47 | −28.51 |
| 8.46E-10 | −27.88 | −28.86 |
| 1.11E-09 | −27.78 | −28.64 |
| 1.17E-09 | −27.82 | −28.65 |
| 1.48E-09 | −27.93 | −28.65 |
| 2.17E-09 | −27.97 | −28.51 |
| 2.18E-09 | −28.13 | −28.67 |
| 2.36E-09 | −28.02 | −28.52 |
| 2.43E-09 | −28.09 | −28.58 |
| 2.53E-09 | −28.00 | −28.47 |
| 2.64E-09 | −28.36 | −28.81 |
| 2.88E-09 | −28.14 | −28.55 |
| 3.27E-09 | −28.04 | −28.39 |
| 3.66E-09 | −28.46 | −28.75 |
| 3.95E-09 | −28.34 | −28.60 |
| 4.01E-09 | −28.19 | −28.44 |
| 4.23E-09 | −28.40 | −28.62 |
| 4.28E-09 | −28.46 | −28.68 |
| 4.52E-09 | −28.44 | −28.63 |
| 4.55E-09 | −28.41 | −28.60 |
| 4.88E-09 | −28.40 | −28.56 |
| 5.70E-09 | −28.65 | −28.73 |
| 6.38E-09 | −28.58 | −28.61 |
| 6.57E-09 | −28.73 | −28.75 |
| Average | −27.98 | −28.61 |
| 1σ* | 0.53 | 0.13 |

* Error to one standard deviation.

good internal precision. Table 2 shows the $\delta^{13}\text{C}$ values for analysis of CO₂ from decane over a range of sample sizes. Before adjusting the $\delta^{13}\text{C}$ values for signal size, the internal precision of the instrument is 0.53‰ for a range of I^{44} between 1.2×10^{-10} to 6.6×10^{-9} A. After correction, the average of the calculated value exactly matches the theoretical value of −28.61‰, while the precision decreases to 0.13‰.

As can be seen from the equations in Figs 3 and 4, the trends for the $\delta^{13}\text{C}$ values of the compounds in the VG mix, phenol and CO₂ are similar. Figure 5 illustrates all data points recorded ($\Delta = \delta^{13}\text{C}_{\text{actual}} - \delta^{13}\text{C}_{\text{true}}$) plotted as a function of the I^{44} intensity. The r^2 value in the equation describing the signal dependence is 0.72. This suggests that an overall correction factor could be used for all data to enhance $\delta^{13}\text{C}$ accuracy and precision over the I^{44} ranges of 2×10^{-10} to 1×10^{-8} A. However, we have found from the data presented that compound specific correction achieves better results.

The data presented here clearly show that the combustion interface performance can not account for the trends outlined in this paper. The water trap, if properly maintained, is of a reliable design and is also an unlikely source of any routine error. With decreasing sample size, any air in the system, and in particular nitrogen, will become a larger proportion of the total sample size. The possible contribution of any incompletely oxidised N₂ in the form of

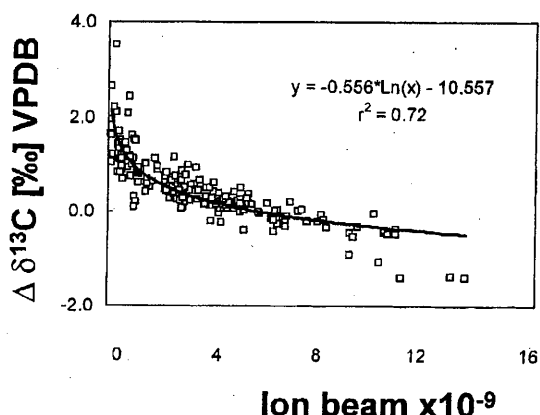


Figure 5. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta \delta^{13}\text{C}_{\text{DIC}}$) for decane, undecane, dodecane and methyl decanoate, phenol and CO_2 from Carrara marble plotted in one graph.

N_2O was therefore of concern. However, any addition of ionised N_2O to the I^{44} beam would produce depleted $\delta^{13}\text{C}$ values with decreasing I^{44} intensity, the opposite of the trend observed. It is interesting to note that the application of a logarithmic correction to sample size dependant $\delta^{13}\text{C}$ values has also been reported for dual inlet measurements.²¹ However, the major difference between continuous flow and dual inlet sample introduction makes the previously published explanation inappropriate. One possible explanation for the signal dependence may be mass diffusion in the IRMS source. There is a flow of CO_2 and helium (He) into the ion source. As the ratio of CO_2 to He changes (i.e. decreasing beam I^{44} strength), there may be an increasing potential to accentuate the diffusional fractionation of mass 44, 45 and 46 and therefore cause variation in residence times of these ions within the source. Further work is needed to elucidate the exact nature of the observed phenomenon.

CONCLUSIONS

A GC has been coupled successfully with two detectors, an MSD and an IRMS. The nature of the flow splits in the instrumental coupling make it desirable to achieve a higher degree of sensitivity than may normally be required when using a routine GC/IRMS. The signal dependent trend observed is similar for all the compounds studied in this paper. However, the signal dependence that occurs from a high sensitivity source tuning can be corrected for. The

results indicate that a general correction equation could be used to correct $\delta^{13}\text{C}$ values for I^{44} intensity. However, where possible, compound specific correction provides the best accuracy and precision. This allows routine continuous flow analysis using a GC/MSD/IRMS system to produce very accurate results to a precision approaching 0.1‰ over two orders of I^{44} magnitude.

Acknowledgements

M. Carey, N. Ogle and J. Hamilton are thanked for their technical assistance. This work was supported by grants from the QUESTOR Industrial Board, Queen's University Belfast, and EPSRC grants L85183 and M26374.

REFERENCES

1. W. A. Brandt, *J. Mass Spectrom.* **31**, 225 (1994).
2. C. R. McKinney, J. M. McCrea and S. Epstein, *Rev. Sci. Instrum.* **21**, 724 (1950).
3. G. St-Jean, Introduction to Stable Isotopes, *The 5th Canadian Continuous Flow Isotope Ratio Mass Spectrometry Workshop*, Ottawa (1988).
4. J. A. Hall, R. M. Kalin, M. J. Larkin, C. C. R. Allen and D. B. Harper, *Org. Geochem.* (in press).
5. P. K. Aggerwal, M. E. Fuller, M. E. Gurgas, J. F. Manning and M. A. Dillon, *Env. Sci. Tech.* **31**, 590 (1991).
6. M. Sano, Y. J. Yotsui, H. Abe and S. Sasaki, *Biomed. Mass Spectrom.* **3**, 1 (1976).
7. D. E. Mathews and J. M. Hayes, *Anal. Chem.* **50**, 1465 (1978).
8. P. A. Eakin, A. E. Fallick and J. Gerk, *Chem. Geol.* **101**, 71 (1992).
9. D. A. Merritt, W. A. Brand and J. M. Hayes, *Org. Geochem.* **21**, 573 (1994).
10. W. Meier-Augenstein, *J. High Res.* **18**, 28 (1995).
11. J. E. Landmeyer, D. A. Vroblesky and F. H. Chapelle, *Env. Sci. Tech.* **30**, 1120 (1996).
12. M. Wittaker, S. T. J. Pollard and T. E. Fallick, *Env. Tech.* **16**, 1009 (1995).
13. W. Meier-Augenstein, W. Brand, G. F. Hoffmann and D. Rating, *Biolog. Mass. Spectrom.* **23**, 376 (1994).
14. H. S. Dempster, B. Sherwood Lollar and S. Feenstra, *Env. Sci. Tech.* **31**, 3193 (1997).
15. C. C. R. Allen, D. R. Boyd, M. J. Larkin, K. A. Reid, N. D. Sharma and K. Wilson, *Appl. Env. Microbiol.* **63**, 151 (1997).
16. R. C. Bayley and S. Dagley, *Biochem. J.* **111**, 111 (1969).
17. K. Rozanski, W. Stichler, R. Gonfiantini, E. M. Scott, R. P. Beukens, B. Kromer and J. Van der Plicht, *Radiocarbon* **34**, 506 (1992).
18. R. M. Kalin, J. A. Hall, U. N. Fitzgerald, Environmental Engineering Research Centre, unpublished data (1998).
19. Micromass UK Ltd., Isochrom GC User Manual, issue 3, pp. 28–29 (1996).
20. J. A. Hall, Environmental Engineering Research Centre, unpublished data (1998).
21. C. M. Scrimgeour, K. Smith and M. J. Rennie, *Biomed. Environ. Mass. Spectrom.* **15**, 369 (1988).

Use of gas chromatography-combustion-isotope ratio mass spectrometry in nutrition and metabolic research

Wolfram Meier-Augenstein

Linking gas chromatography via an on-line combustion interface to isotope ratio mass spectrometry has opened the door to high-precision compound-specific isotope analysis. For this reason, gas chromatography-combustion-isotope ratio mass spectrometry is now increasingly employed in metabolic and nutritional research because it offers a reliable and risk-free alternative to the use of radioactive tracers. *Curr Opin Clin Nutr Metab Care* 2:000-000. © 1999 Lippincott Williams & Wilkins.

Correspondence to Wolfram Meier-Augenstein, CChem MRSC, Senior Research Fellow, University of Dundee, Department of Anatomy and Physiology, OMS, Small's Wynd, Dundee, DD1 4HN, UK
Tel: +44 0 1382 345124; fax: +44 0 1382 345514; e-mail: w.meieraugenstein@dundee.ac.uk

Current Opinion in Clinical Nutrition and Metabolic Care 1999, 2:000-000

Abbreviations

| | |
|-----------|---|
| APE | atom% excess |
| BCAA | branched-chain amino acids |
| GC-C-IRMS | gas chromatography-combustion-isotope ratio mass spectrometry |
| CSIA | compound-specific isotope analysis |
| GC-MS | gas chromatography-mass spectrometry |
| IRMS | isotope ratio mass spectrometry |
| SIM | selected ion monitoring mode |

© 1999 Lippincott Williams & Wilkins
1363-1950

Introduction

One of the reasons for the increasing appeal of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is the fact that, as a result of this technology, stable isotope labelled tracers have become a viable alternative to radioactive tracers, a fact that is of particular relevance when dealing with paediatric patients. With the exception of deuterium (^2H) when used in high concentrations, heavier stable isotopes such as ^{13}C , ^{15}N and ^{18}O do not cause any adverse physiological effects, even at high enrichment levels [1]. Isotope ratio mass spectrometry (IRMS) is an analytical mass spectroscopic technology, for it provides quantitative information rather than structural information on a given compound. The application of high-precision compound-specific isotope analysis (CSIA) in nutrition and metabolic research can be roughly divided into two areas. One area of application is concerned with studies to measure the rate of intermediary metabolism, the other aims to trace metabolic pathways using labelled precursor compounds at high enrichment levels to improve detection limits. This article aims to present an overview of the work in these areas during the past 4 years. Because this is the first time that GC-C-IRMS is presented in this forum, some emphasis is also placed on the methodological aspects and constraints of this hyphenated technique.

Practical issues concerning gas chromatography-combustion-isotope ratio mass spectrometry

In contrast to organic mass spectrometers that yield structural information by scanning a mass range over several hundred Daltons for characteristic fragment ions, IRMS instruments achieve a highly accurate and precise measurement of isotopic abundance at the expense of the flexibility of scanning mass spectrometers (Table 1). Because gas chromatography-mass spectrometry (GC-MS) can be used to measure stable isotope enrichment, the question arises as to why one should embrace GC-C-IRMS. Scanning mass spectrometers use a single detector and therefore cannot simultaneously detect particular isotope pairs for isotope ratio measurement. For isotope ratio measurement, the mass spectrometer is best operated in selected ion monitoring mode (SIM) to optimize the sensitivity to selected masses. Even in SIM mode, the limited accuracy and precision of such isotope ratio measurements impose a minimum working enrichment for ^{13}C and ^{15}N of at least 0.5 atom% excess (APE) [2,3]. In other words, organic mass spectrometry cannot

provide reliable quantitative information in cases in which a low turnover or low rate of incorporation results in isotopic enrichment of less than 0.5 APE.

In contrast, GC-C-IRMS can measure isotopic composition at low enrichment and natural abundance levels. This means that minute variations in very small amounts of the heavier isotope are detected in the presence of large amounts of the lighter isotope. Because the small variations of the heavier isotope habitually measured by IRMS are of the order of -0.07 to $+1.09$ APE, the δ -notation in units of per mil (‰) has been adopted to report changes in isotopic abundance as a per mil deviation compared with a designated isotopic standard:

$$\delta_s = [(R_s - R_{std})/R_{std}] \times 1000 \text{ ‰}$$

where R_s is the measured isotope ratio for the sample and R_{std} is the measured isotope ratio for the standard. To give a convenient rule-of-thumb approximation, in the δ -notation, a ^{13}C enrichment in the range of -0.033 to $+0.0549$ APE corresponds to a $\delta^{13}\text{C}$ value range of -30‰ to $+50\text{‰}$. A change of $+1\text{‰}$ is approximately equivalent to a change of 0.001 APE and 0.0003 APE for ^{13}C and ^{15}N , respectively.

The sensitivity of GC-C-IRMS is such that tracer/tracee (mol/mol) ratios down to 10^{-5} can be reliably detected [4]; in the same review, Brenna *et al.* also provide an in-depth discussion of notations and elementary calculations

such as mass balance and pool mixing equations.

As a result of its high sensitivity, GC-C-IRMS depends on careful sample preparation and high-resolution capillary gas chromatography [5**]. Demands on sample size, sample derivatization, quality of gas chromatography separation, interface design and isotopic calibration have been discussed in a number of reviews [2,3,5**,6-8,9**].

High-precision compound-specific isotope analysis of ^{13}C isotopic abundance

The great clinical and scientific potential of ^{13}C tracer techniques for nutritional and metabolic research in paediatric patients has been discussed recently [10**]. Several comparative studies have demonstrated the considerable advantages of GC-C-IRMS in nutritional and metabolic research. Pont *et al.* [11] measured ^{13}C enrichment in the cholesterol of rabbit low-density lipoprotein after the injection of 3 mg of $[3,4-^{13}\text{C}]\text{cholesterol}$. They compared the accuracy and precision, detection limits and dynamic range (ranging from -22 to $+760$) of GC-C-IRMS and GC-MS measurements and found GC-C-IRMS to be more accurate and reproducible, especially at lower enrichment levels. Highly linear calibration curves for ^{13}C enrichment were also reported from a study with branched-chain amino acids (BCAA) [12]. In that study, slopes of 0.98 and 1.04 were observed for enrichment ranges $0-0.14$ APE and $0-$

Table 1. Typical features and specifications of mass spectrometry systems used for stable isotope analysis

| | GC-MS | Elemental analyser- IRMS | GC-C-IRMS |
|--|--|---|--|
| Sample introduction | Injection of liquid (or gaseous) sample matrix on to GC column | Solid (and dry) sample in tin capsules | Injection of liquid (or gaseous) sample matrix on to GC column |
| Sample separation | Yes, by gas chromatography | No | Yes, by gas chromatography |
| Sample manipulation before mass spectroscopic analysis | None | Combustion/reduction of sample into CO_2 and N_2 in the elemental analyser | Combustion/reduction of compounds into CO_2 and N_2 in the interface |
| Interface | Heated transfer capillary directly connected to ion source | Transfer capillary with open split | Capillary, incorporating wide bore combustion/reduction furnaces |
| Mass analysers | Quadrupole | Magnet | Magnet |
| Detector | One electron multiplier | Triple Faraday cup collector | Triple Faraday cup collector |
| Mode of charged mass | SIM, switching between e.g. M^+ and $[\text{M}+1]^+$ ($[\text{M}+1]^+/ \text{M}^+$ ratios are calculated on the basis of measured ion current) | Simultaneous detection of particles with three adjacent masses, e.g. m/z 44, 45 and 46 for $^{12}\text{C}^{16}\text{O}_2$, $^{13}\text{C}^{16}\text{O}_2$ and $^{12}\text{C}^{18}\text{O}^{16}\text{O}$, respectively | Simultaneous detection of particles with three adjacent masses, e.g. m/z 28, 29 and 30 for $^{14}\text{N}^{14}\text{N}$, $^{14}\text{N}^{15}\text{N}$ and $^{15}\text{N}^{15}\text{N}$, respectively |
| Detection | | -0.1 – $+2.0$ | -0.1 – $+2.0$ |
| Measureable enrichment range in [APE] | $+0.5$ – 100^a | | |
| Sample size requirement | $\leq 1 \text{ pmol}$ | 0.1 – $5 \text{ } \mu\text{mol}^b$ | 0.1 – 5 nmol^c |
| CSIA | Yes, with a precision of $0.05 \text{ atom}\%$ | No ^d | Yes, with a precision of $0.0002 \text{ atom}\%$ |

^aUsing a multiply labelled tracer (e.g. $[^2\text{H}_5]\text{phenylalanine}$) and measuring $[\text{M}+n]^+/ \text{M}^+$ ratios (e.g. $[\text{M}+5]^+/ \text{M}^+$), enrichments down to 0.2 APE can be reliably detected.

^bThe required sample size depends on the type of isotope analysis. Analysis of ^{13}C enrichment requires only small amounts of material because of the high abundance of carbon in organic compounds. The opposite is true for the analysis of ^{15}N enrichment, because of the low abundance of nitrogen in organic compounds. Furthermore, for compounds containing only one atom of nitrogen, two moleequivalents of compound have to be combusted to generate one moleequivalent of N_2 .

^cIn addition to the considerations mentioned above, it should be noted that compound-specific isotope analysis (CSIA) by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) requires the injection of 0.1 – 5 nmol per individual compound to be analysed.

^dUsing elemental analyser-IRMS, CSIA is only possible for off-line isolated and purified compounds. Here, the precision is $0.001 \text{ atom}\%$. APE, Atom% excess; GC-MS, gas chromatography-mass spectrometry; SIM, selected ion monitoring mode.

8 APE, respectively. Parker *et al.* [13] showed that GC-C-IRMS permits the use of low doses of β -[U- ^{13}C]carotene (≤ 2 mg), which do not perturb endogenous pool sizes of β -carotene or retinol. Employing ^{13}C tracers at low levels of enrichment, reliable data were obtained in studies of the kinetics of glycoprotein neutral sugars [14] and urea [15]. Bunt *et al.* [16*] used [U- ^{13}C]glucose as a precursor to obtain data on endogenous surfactant production and turnover by measuring ^{13}C -enrichment of palmitic acid in phosphatidylcholine palmitate. GC-C-IRMS was also used to determine the range of natural isotope abundance of ^{13}C in six serum fatty acids from humans on a controlled diet [17].

Compound-specific isotope analysis of ^{13}C labelled fatty acids

The high sensitivity of GC-C-IRMS has been increasingly exploited in metabolic studies investigating the turnover, incorporation and synthesis processes of fatty acids *in vivo*, which could only previously be investigated either with stable isotope tracers at high enrichment levels using GC-MS, or not at all [18].

GC-C-IRMS, capable of measuring naturally occurring differences in isotope ratios, determined the extent to which neonates and very low birthweight premature infants could synthesize arachidonic acid, which is essential for their growing tissues, from dietary fatty acids. Demmelmair *et al.* [19] showed that in neonates fed on a phenylalanine-free diet, on average 23% of free plasma arachidonic acid on study day 4 originated from infantile linoleic acid conversion. Carnielli *et al.* [20] added linoleic acid and linolenic acid, both ^{13}C -labelled, to the formula diet that was administered continuously for 48 h (birthweight 1.17 ± 0.12 kg; gestational age 28.4 ± 1.3 weeks). The authors demonstrated that both tracers were rapidly incorporated into plasma phospholipids and that their metabolic products including arachidonic acid and docosahexaenoic acid became highly enriched with ^{13}C . The incorporation of ^{13}C -octanoic acid into plasma triglycerides (10% of the enrichment of the diet), noticeably into myristic and palmitic acid, by very low-weight preterm infants was reported earlier by the same group [21].

The majority of ^{13}C tracer studies published have dealt with various aspects of fatty acid biochemistry. The bioequivalence of dietary α -linolenic and docosahexaenoic acid as substrates for brain and retinal n3 fatty acid accretion during the brain growth spurt [22,23*], metabolism of triglycerides [24], transport and turnover of free saturated [25,26,27*] and unsaturated fatty acids [28–30,31*]. While studying the metabolism of ^{13}C -labelled polyunsaturated fatty acids by ^{13}C -nuclear magnetic resonance, using GC-C-IRMS, Cunnane *et al.* [32] found low levels of ^{13}C -labelled γ -linolenic acid

in the brain phospholipids of suckling rat pups that could not be detected by ^{13}C -nuclear magnetic resonance.

Unlike GC-MS, in which increasing the amount of label has no general effect on detection limits, in GC-C-IRMS, increasing label enrichment in precursor compounds produces significantly improved detection limits. Taking advantage of this increased traceability, Rhee *et al.* [33] quantified the desaturation of [U- ^{13}C]18:0 and [U- ^{13}C]16:0 in whole plasma and lipoprotein of adults after the administration of 30 mg oral doses (< 0.5 mg/kg). Su and Brenna [34*] reported the simultaneous measurement of desaturase activities by monitoring the increase in product from the reactions [U- ^{13}C]18:2n6 \rightarrow [U- ^{13}C]18:3n6 and [U- ^{13}C]16:0 \rightarrow [U- ^{13}C]16:1n7, respectively.

Using [U- ^{13}C] α -linolenic acid, Sheaff *et al.* [35] were able to demonstrate that high dietary levels of linoleic acid did not depress the conversion of α -linolenate into docosahexaenoate. Three years later, Menard *et al.* [36*] showed that [U- ^{13}C] α -linolenic acid is not solely a source of docosahexaenoic acid. They found that, owing to a high rate of β -oxidation and carbon recycling, [U- ^{13}C] α -linolenate was utilized in the de-novo synthesis of cholesterol and palmitate.

Compound-specific isotope analysis of ^{13}C -labelled amino acids

In spite of the wide usage that GC-C-IRMS enjoys in research concerned with fatty acid and surfactant metabolism, most research groups interested in amino acid metabolism and protein turnover still seem to favour traditional GC-MS methods such as SIM in conjunction with poly-deuterated tracers or tracers highly enriched in ^{13}C . This may be because GC-MS methods cannot easily be adapted to suit the particular needs of GC-C-IRMS. As mentioned above, the nature of the derivatization agent can influence the accuracy and precision of CSIA, because it will dilute ^{13}C enrichment and can adversely affect chromatographic separation and the efficiency of the combustion catalyst (cf. Table 2) [5*,7,9**].

Seemingly in response to these difficulties, most groups using GC-C-IRMS to study turnover and synthesis rates of various proteins have adopted a strategy whereby a target amino acid is identified that will act as a marker providing unambiguous and quantitative information about the system under investigation. This strategy included the development of a sample preparation protocol tailor-made for the particular amino acid allowing quick and reliable CSIA.

In this way, L-[^{13}C -1]valine was employed to measure in-vivo secretion rates of very low density lipoprotein-

Table 2. Overview of typical analytical procedures used in metabolic and nutritional research involving stable isotopes and their caveats

| Compound class | Derivatization | Typical GC conditions ^a | Caveats |
|--|---|---|---|
| Long-chain alcohols and sterols (e.g. cholesterol) | TMS | CP-Sil 5: 50°C (2 min) to 200°C at 40°/min, 200–320°C at 3°/min | None |
| Fatty acids | Methyl ester using methanol/ BF ₃ | CP-Sil 8/CP-Sil 19: 120°C (4 min) to 280°C at 4°/min | None |
| Hydroxy/amino/keto-carboxylic acids | TMS or tBDMS | CP-Sil 8: 80°C (5 min) to 150°C at 2°/min, 150–220°C at 3.5°/min; 20°/min to 300°C | TMS: multiple derivatives for same compound ^b tBDMS: excessive carbon load ^c Risk of non-quantitative derivatization ^d |
| Hydroxy/amino.keto-carboxylic acids | Ethylchloroformates | CP-Sil 24: 60°C (3 min) to 300°C at 6°/min | Risk of non-quantitative derivatization ^d |
| Hydroxy/amino/keto-carboxylic acids | Acetyl, methylates | CP-Sil 19: 70°C (5 min) to 220°C at 5°/min | None |
| Amino acids | TMS or tBDMS | CP-Sil 8: 50°C (5 min) to 150°C at 6°/min, 150–300°C at 12°/min | TMS: multiple derivatives for same compound ^b tBDMS: excessive carbon load ^c Poisoning of combustion catalyst ^e |
| Amino acids | TFA, methylates TFA, iso-propylates | CP-Sil 19: 70°C (5 min) to 180°C at 3.5°/min, 180–230°C at 5°/min; 10°/min to 270°C | |
| Amino acids | Ethylchloroformates, methylchloroformates | CP-Sil 19: 60°C (3 min) to 100°C at 5°/min, 100–300°C at 10°/min | Risk of non-quantitative derivatization ^d |
| Amino acids | N-Pivaloyl, iso-propylates | CP-Sil 8: 70°C (1 min) to 200°C at 3°/min, 220–300°C at 10°/min | None |
| Amino acids | N-Acetyl, propylates | CP-Sil 19: 70°C (5 min) to 200°C at 4°/min, 200–300°C at 6°/min | None |

^aThe content of this column should be read as generalized guidelines providing a starting point for the reader to resolve individual analytical tasks. Information provided is choice of gas chromatography (GC) column (=stationary phase) and temperature programme. CP-Sil is a proprietary name of CHROMPACK International BV (Middelburg, the Netherlands) and has been chosen as an example because it is widely recognized.

^bAmbiguity of results; a problem for gas chromatography-mass spectrometry (GC-MS) as well as gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

^cStrong dilution of ¹³C enrichment; this affects only GC-C-IRMS measurements. It is, however, only a problem when the measurement cannot be compared against the background or baseline ^{δ13}C value of an authentic sample.

^dPotential loss of information (GC-MS and GC-C-IRMS).

^eA serious problem for GC-C-IRMS; leads ultimately to incomplete combustion and can negatively affect the accuracy and precision of ¹³C and ¹⁵N isotope abundance measurements.

TFA, Trifluoroacetyl; TMS, Trimethylsilyl; tBDMS, tert-Butyldimethylsilyl.

apolipoprotein B100 in humans [37*], synthesis rates of α_2 -macroglobulin in nephrotic patients [38*] and the protein fractional synthetic rate in skeletal muscle [39*] and plasma albumin [40*].

In the same way, other groups used L-[¹³C-1]leucine to measure the fractional synthesis rate of mixed muscle protein, myosin heavy chain and actin in human skeletal muscle [41*,42], the metabolism of human apolipoprotein B [43] and protein synthesis in patients suffering from rectal cancer [44,45].

To measure collagen synthesis in adult humans, Rennie *et al.* [3] used L-[¹³C-1]proline with an enrichment of 30 mol% excess as the marker amino acid. The usefulness of proline as a tracer lies in the phenomenon of post-translational hydroxylation of collagen-bound proline into 4-hydroxyproline and the fact that all protein-bound 4-hydroxyproline is in collagen.

Menand *et al.* [46] described a method to determine low enrichment levels of free L-[¹³C-1]glutamine in plasma, and Meier-Augenstein *et al.* [12] reported a method to measure [¹³C-1]-labelled BCAA L-valine, L-leucine and L-isoleucine, as well as their transamination products in

human plasma after oral bolus administration. The authors demonstrated that this method could be used to measure whole-body BCAA oxidation in patients suffering from maple syrup urinary disease [12].

High-precision compound-specific isotope analysis of ¹⁵N-labelled amino acids

Published work on studies of amino acid and protein turnover using ¹⁵N isotopic abundance measurements by GC-C-IRMS are still few and far between. This is quite probably a reflection of the analytical challenges associated with high precision CSIA of ¹⁵N isotopic abundance in amino acids. First, the low concentration of nitrogen in amino acids, for amino acids contain two to 11 times more carbon than nitrogen. Second, ^{δ15}N measurements require N₂, which at least doubles the sample size requirement because two molequivalents of amino acids are needed for the production of one molequivalent of N₂. Non-quantitative sample conversion and reduced sensitivity of the IRMS ion source for N₂ (compared with CO₂) increase sample size requirements further, by a factor of five compared with CSIA of ¹³C-labelled compounds. Finally, even small amounts of atmospheric gas leaks into the GC-C-IRMS instrument result in a high N₂ background level.

However, CSIA of nitrogenous compounds such as amino acids yields new and unexpected insights into the metabolism of organic nitrogen. For example, a study of plasma albumin synthesis using L-[^{13}C -1, ^{15}N]alanine and L-[^{13}C -1, ^{15}N]leucine showed that both amino acids underwent transamination (and re-amination with nitrogen from the body's nitrogen pool) before their incorporation into plasma protein [3]. The observed enrichment ratios of $^{15}\text{N}/^{13}\text{C}$ found in plasma albumin were 1:3.18 and 1:2.29 for alanine and leucine, respectively. A cross-over of ^{13}C could be ruled out because $\delta^{13}\text{C}$ -values for simultaneously administered L-[^{13}C -1]phenylalanine remained at baseline level throughout the course of the study.

Preston *et al.* [47] measured protein synthesis using [^{15}N]glycine to study the effect of ibuprofen on the plasma concentration of acute-phase proteins in patients with colonic cancer. The authors found that ibuprofen caused a significant reduction of all five acute-phase proteins (fibrinogen, C-reactive protein, caeruloplasmin, α_1 -antitrypsin and α_1 -acid glycoprotein) after 3 days of oral ibuprofen.

Monitoring $\delta^{15}\text{N}$ -values of free plasma amino acids from fasting human subjects, Metges and Petzke [48] made the intriguing observation of consistently low levels of ^{15}N abundance ($\delta^{15}\text{N} < 0.0\text{‰}$ versus air) in the amino acids threonine and phenylalanine. The $\delta^{15}\text{N}$ values of all the other amino acids were positive ($\delta^{15}\text{N} > 0.0$ versus air). In subsequent studies that involved the administration of oral bolus doses of [$^{15}\text{N}_2$]urea, Petzke *et al.* [49] found that incorporation of ^{15}N from this source into plasma protein amino acids was consistently low for lysine, histidine, proline and phenylalanine. On the basis of the results of a widened study combining the administration of [$^{15}\text{N}_2$]urea with the ingestion of lactic acid bacteria, Petzke *et al.* [50] hypothesized that these low levels of ^{15}N incorporation suggest only limited or no participation in the transamination reactions of those amino acids.

Conclusion

High-precision CSIA by GC-C-IRMS of organic compounds at natural abundance and low enrichment levels is a powerful tool that provides new insights as well as quantitative information on human metabolism. GC-C-IRMS is a unique tool that can provide answers to biochemical and physiological questions that cannot be obtained by any other analytical instrumentation.

Acknowledgements

The author gratefully acknowledges financial support through the Dietmar-Hopp Foundation for Medical Research and Studies (Walldorf, Germany). Many thanks are due to Dr Helen F. Kemp (University of Dundee) and Professor J. Thomas Brenna (Cornell University) for critical reading of the manuscript.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

- 1 Koletzko B, Sauerwald T, Demmelmair H. Safety of stable isotope use. *Eur J Pediatr* 1997; 156:S12-S17.
- 2 Preston T, Slater C. Mass-spectrometric analysis of stable-isotope-labeled amino-acid tracers. *Proc Nutr Soc* 1994; 53:363-372.
- 3 Rennie MJ, Meier-Augenstein W, Watt PW, Patel A, Begley IS, Scrimgeour CM. Use of continuous-flow combustion ms in studies of human metabolism. *Biochem Soc Trans* 1996; 24:927-932.
- 4 Brenna JT, Corso TN, Tobias HJ, Caimi RJ. High-precision continuous-flow isotope ratio mass spectrometry. *Mass Spectrom Rev* 1997; 16:227-258.
- One of the best general review articles on IRMS published in recent years. Includes a section dedicated to aspects important for tracer studies such as mass balance, pool mixing and dilution space.
- 5 Meier-Augenstein W. Applied gas chromatography coupled to isotope ratio mass spectrometry. *J Chromatogr A* 1999; 842:351-371.
- This most recent review on GC-C-IRMS gives a comprehensive overview of its wide spectrum of applications. It also provides detailed information on sample preparation, gas chromatographic methods and isotopic calibration.
- 6 Brand WA. High precision isotope ratio monitoring techniques in mass spectrometry. *J Mass Spectrom* 1996; 31:225-235.
- 7 Meier-Augenstein W. The chromatographic side of isotope ratio mass spectrometry-pitfalls and answers. *LC/GC-Mag Sep Sci* 1997; 15:244-253.
- 8 Ellis L, Fincannon AL. Analytical improvements in irm-GC/MS analyses: advanced techniques in tube furnace design and sample preparation. *Org Geochem* 1998; 29:1101-1117.
- 9 Metges CC, Petzke KJ. The use of GC-C-IRMS for the analysis of stable isotope enrichment in nitrogenous compounds. In: *Methods of investigation of amino acid and protein metabolism*, chapter 7. El-Khoury AE (editor). Boca Raton, Florida: CRC Press LLC; 1999; pp. 121-134.
- The only review dedicated to aspects of stable isotope analysis of nitrogenous compounds.
- 10 Koletzko B, Demmelmair H, Hartl W, Kindermann A, Koletzko S, Sauerwald T, Sztanyl P. The use of stable isotope techniques for nutritional and metabolic research in paediatrics. *Early Hum Dev* 1998; 53:S77-S97.
- A very useful reference article, especially regarding questions of safety and dosage of stable isotope tracers used in nutritional and metabolic research.
- 11 Pont F, Duvillard L, Maugeais C, Athias A, Persegol L, Gambert P, Verges B. Isotope ratio mass spectrometry, compared with conventional mass spectrometry in kinetic studies at low and high enrichment levels: application to lipoprotein kinetics. *Anal Biochem* 1997; 248:277-287.
- 12 Meier-Augenstein W, Rating D, Hoffmann GF, Wendel U, Matthiesen U, Schade-Waldt P. Determination of ^{13}C enrichment by conventional GC-MS and GC-(MS)-C-IRMS. *Isotopes Environ Health Stud* 1995; 31:261-266.
- 13 Parker RS, Brenna JT, Swanson JE, Goodman KJ, Marmor B. Assessing metabolism of beta-[^{13}C]carotene using high-precision isotope ratio mass spectrometry. *Methods Enzymol* 1997; 282:130-140.
- 14 Rambal C, Pachiaudi C, Normand S, Riou JP, Louisot P, Martin A. Differential incorporation of c-13 label from dietary glucose into neutral sugars of rat intestine macromolecules. *Ann Nutr Metab* 1995; 39:143-151.
- 15 Kloppenburg WD, Wolthers BG, Stellaard F, Elzinga H, Tepper T, deJong PE, Huisman RM. Determination of urea kinetics by isotope dilution with [^{13}C]urea and gas chromatography isotope ratio mass spectrometry (GC-IRMS) analysis. *Clin Sci* 1997; 93:73-80.
- 16 Bunt JEH, Zimmermann LJ, Wattimena JLD, vanBeek RH, Sauer PJ, Camielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotopes. *Am J Respir Crit Care Med* 1998; 157:810-814.
- An in-vivo study of surfactant phosphatidylcholine palmitate in which [^{13}C]glucose was used as a precursor, infused on the first day of life of preterm infants.
- 17 Rhee SK, Reed RG, Brenna JT. Fatty acid carbon isotope ratios in humans on controlled diets. *Lipids* 1997; 32:1257-1263.
- 18 Brenna JT. Use of stable isotopes to study fatty acid and lipoprotein metabolism in man. *Prostaglandins Leukotrienes Essent Fatty Acids* 1997; 57:467-472.
- 19 Demmelmair H, Vonschenck U, Behrendt E, Sauerwald T, Koletzko B. Estimation of arachidonic-acid synthesis in full-term neonates using natural variation of c-13 content. *J Pediatr Gastroenterol Nutr* 1995; 21:31-36.

- 20 Carnielli VP, Wattimena DJL, Luijendijk IHT, Boerlage A, Degenhart HJ, Sauer PJJ. The very-low-birth-weight premature-infant is capable of synthesizing arachidonic and docosahexaenoic acids from linoleic and linolenic acids. *Pediatr Res* 1996; 40:169-174.
 - 21 Carnielli VP, Sulkers EJ, Moretti C, Wattimena JLD, Vangoudoever JB, Degenhart HJ, et al. Conversion of octanoic-acid into long-chain saturated fatty-acids in premature-infants fed a formula containing medium-chain triglycerides. *Metabolism* 1994; 43:1287-1292.
 - 22 Sheaff Greiner RC, Winter J, Nathanielsz PW, Brenna JT. Brain docosahexaenoate accretion in fetal baboons: bioequivalence of dietary alpha-linolenic and docosahexaenoic acids. *Pediatr Res* 1997; 42:826-834.
 - 23 Su HM, Bernardo L, Mirmiran M, Ma XH, Corso TN, Nathanielsz PW, Brenna JT. Bioequivalence of dietary alpha-linolenic and docosahexaenoic acids as sources of docosahexaenoate accretion in brain and associated organs of neonatal baboons. *Pediatr Res* 1999; 45:87-93.
- The latest results in a series of studies that investigated the role of dietary α -linolenic and docosahexaenoic acids as substrates for brain and retinal n-3 fatty acid accretion.
- 24 Binnert C, Laville M, Pachiaudi C, Rigalleau V, Beylot M. Use of gas-chromatography isotope ratio-mass spectrometry to study triglyceride-metabolism in humans. *Lipids* 1995; 30:869-873.
 - 25 Stolinski M, Murphy JL, Jones AE, Jackson AA, Wootton SA. Stable-isotope method for determining the gastrointestinal handling of [1-C-13]palmitic acid. *Lipids* 1997; 32:337-340.
 - 26 Guo ZK, Nielsen S, Burguera B, Jensen MD. Free fatty acid turnover measured using ultra low doses of [U-C-13]palmitate. *J Lipid Res* 1997; 38:1888-1895.
 - 27 Guo ZK, Jensen MD. Intramuscular fatty acid metabolism evaluated with stable isotopic tracers. *J Appl Physiol* 1998; 84:1674-1679.
- A pilot study that used [U-¹³C]palmitate and [¹³C-1]oleate to measure intramuscular metabolism of non-esterified fatty acids and triglycerides.
- 28 Croset M, Brossard N, Pachiaudi C, Normand S, Lecerf J, Chirouze V, et al. In-vivo compartmental metabolism of c-13 docosahexaenoic acid, studied by gas-chromatography combustion isotope ratio mass-spectrometry. *Lipids* 1996; 31:S109-S115.
 - 29 Demmelmair H, Sauerwald T, Koletzko B, Richter T. New insights into lipid and fatty acid metabolism via stable isotopes. *Eur J Pediatr* 1997; 156:S70-S74.
 - 30 Brossard N, Croset M, Normand S, Pousin J, Lecerf J, Laville M, et al. Human plasma albumin transports [C-13]docosahexaenoic acid in two lipid forms to blood cells. *J Lipid Res* 1997; 38:1571-1582.
 - 31 Demmelmair H, Baumheuer M, Koletzko B, Dokoupil K, Kratl G. Metabolism of [U-C-13]-labeled linoleic acid in lactating women. *J Lipid Res* 1998; 39:1389-1396.
- By administering [U-¹³C]linolenic acid to lactating women, the origin of long-chain polyunsaturated fatty acids in breast milk was partly identified. Approximately 30% of milk linolenic acid is derived from the diet by direct transfer.
- 32 Cunnane SC, Moine G, Likhodii SS, Vogt J, Corso TN, Brenna JT, et al. [3-C-13] gamma-linolenic acid: a new probe for C-13 nuclear magnetic resonance studies of arachidonic acid synthesis in the suckling rat. *Lipids* 1997; 32:211-217.
 - 33 Rhee SK, Kayani AJ, Brenna JT. Desaturation and interconversion of dietary stearic and palmitic acids in human plasma and lipoproteins. *Am J Clin Nutr* 1997; 65:451-458.
 - 34 Su HM, Brenna JT. Simultaneous measurement of desaturase activities using stable isotope tracers or a nontracer method. *Anal Biochem* 1998; 261:43-50.
- Microsomal Δ^6 - and Δ^9 -desaturase activities were quantified by measuring the increase in product concentration. The pH and temperature dependency of these reactions were also monitored.
- 35 Sheaff RC, Su HM, Keswick LA, Brenna JT. Conversion of alpha-linolenate to docosahexaenoate is not depressed by high dietary levels of linoleate in young rats-tracer evidence using high-precision mass-spectrometry. *J Lipid Res* 1995; 36:998-1008.
 - 36 Menard CR, Goodman KJ, Corso TN, Brenna JT, Cunnane SC. Recycling of carbon into lipids synthesized de novo is a quantitatively important pathway of alpha-[U-C-13]linolenate utilization in the developing rat brain. *J Neurochem* 1998; 71:2151-2158.
- This study showed that, because of a high rate of β -oxidation and carbon recycling, α -linolenate cannot be depended upon as sole source of docosahexaenoate for the developing brain.
- 37 deSainvanderVelden MGM, Rabelink TJ, Gadellaa MM, Elzinga H, Reijngoud DJ, Kuipers F, Stellaard F. In vivo determination of very-low-density lipoprotein-apolipoprotein B100 secretion rates in humans with a low dose of L-[1-C-13]valine and isotope ratio mass spectrometry. *Anal Biochem* 1998; 265:308-312.
- Fractional secretion rate of very low-density lipoprotein-apolipoprotein B100 was measured using a minimal infusion of L-[¹³C-1]valine in conjunction with GC-C-IRMS. The obtained secretion rate of 0.002 ± 0.09 pools/h was in good agreement with that obtained by traditional GC-MS methods (0.23 ± 0.07 pools/h).
- 38 deSainvanderVelden MGM, Rabelink TJ, Reijngoud DJ, Gadellaa MM, Voorbij HAM, Stellaard F, Kaysen GA. Plasma alpha(2) macroglobulin is increased in nephrotic patients as a result of increased synthesis alone. *Kidney Int* 1998; 54:530-535.
- Combining immunoprecipitation and sodium dodecylsulfate-polyacrylamide gel electrophoresis with low dosage stable isotope techniques, this study demonstrated that the increased plasma α_2 macroglobulin concentration in nephrotic patients is a result of increased synthesis alone.
- 39 Reijngoud DJ, Hellstern G, Elzinga H, deSainvanderVelden MGM, Okken A, Stellaard F. Determination of low isotopic enrichment of L-[1-C-13]valine by gas chromatography combustion isotope ratio mass spectrometry: a robust method for measuring protein fractional synthetic rates in vivo. *J Mass Spectrom* 1998; 33:621-626.
- A comprehensive GC-C-IRMS method is described to measure protein fractional synthesis rates using L-[¹³C-1]valine as the tracer. The tracer detection limit of this method was 0.0002 (tracer/tracee ratio in mol/mol).
- 40 Kulik W, Meesterburrie JAN, Jakobs C, deMeer K. Determination of delta C-13 values of valine in protein hydrolysate by gas chromatography combustion isotope ratio mass spectrometry. *J Chromatogr B* 1998; 710:37-47.
- A methodological study that demonstrated high linearity of $\Delta^{13}\text{C}$ measurements over a wide dynamic range of enrichment (0-1.5 mol% excess).
- 41 Hasten DL, Morris GS, Ramanadham S, Yarasheski KE. Isolation of human skeletal muscle myosin heavy chain and actin for measurement of fractional synthesis rates. *Am J Physiol Endocrinol Metab* 1998; 38:E1092-E1099.
- A simple yet more refined approach was reported for in-vivo studies of muscle contractile protein metabolism. This approach permitted not only the fractional synthesis rate for mixed muscle protein but also myosin heavy chain and actin from small (60-80 mg) human skeletal muscle samples.
- 42 Balagopal P, Ford GC, Ebenstein DB, Nadeau DA, Nair KS. Mass spectrometric methods for determination of [C-13] leucine enrichment in human muscle protein. *Anal Biochem* 1996; 239:77-85.
 - 43 Demant T, Packard CJ, Demmelmair H, Stewart P, Bedynek A, Bedford D, et al. Sensitive methods to study human apolipoprotein-b metabolism using stable isotope-labeled amino-acids. *Am J Physiol Endocrinol Metab* 1996; 33:E1022-E1036.
 - 44 Hartl WH, Demmelmair H, Jauch KW, Schmidt HL, Koletzko B, Schildberg FW. Determination of protein synthesis in human rectal cancer in situ by continuous [1-C-13]leucine infusion. *Am J Physiol Endocrinol Metab* 1997; 35:E796-E802.
 - 45 Hartl WH, Demmelmair H, Jauch KW, Koletzko B, Schildberg FW. Effect of glucagon on protein synthesis in human rectal cancer in situ. *Ann Surg* 1998; 227:390-397.
 - 46 Menand C, Pouteau E, Marchini S, Maugere P, Krempf M, Darmaun D. Determination of low C-13-glutamine enrichments using gas chromatography combustion-isotope ratio mass spectrometry. *J Mass Spectrom* 1997; 32:1094-1099.
 - 47 Preston T, Fearon KCH, McMillan DC, Winstanley FP, Slater C, Shenkin A, Carter DC. Effect of ibuprofen on the acute-phase response and protein-metabolism in patients with cancer and weight-loss. *Br J Surg* 1995; 82:229-234.
 - 48 Metges CC, Petzke KJ. Measurement of N-15/N-14 isotopic composition in individual plasma free amino acids of human adults at natural abundance by gas chromatography combustion isotope ratio mass spectrometry. *Anal Biochem* 1997; 247:158-164.
 - 49 Petzke KJ, Korkushko OV, Semesko TM, Metges CC. N-isotopic composition in human plasma protein amino acids at natural abundance level and after a single [N-15(2)]urea administration measured by GC-C-IRMS. *Isotopes Environ Health Stud* 1997; 33:267-275.
 - 50 Petzke KJ, Grigorov JG, Korkushko OV, Kovalenko NK, Semesko TG, Metges CC. Incorporation of urea nitrogen into fecal protein and plasma protein amino acids in elderly human volunteers after ingestion of lactic acid bacteria. *Z Ernahrungswiss* 1998; 37:368-375.
- The beneficial health effects of fermented milk products may be linked to the incorporation of urea nitrogen into plasma protein amino acids as a result of the metabolic activity of ingested lactic acid bacteria.

Department of Communications, Information Technology and the Arts

Anti-Doping Research Program

Progress Report

March 2004

**Project: Statistical Population Studies to Support New Analytical
Methodologies using the EPO2000 Project Urine Samples.**

Investigators

R. Kazlauskas, G. J. Trout, C. Howe and J. Rogerson (Australian Sports Drug
Testing Laboratory)

SUMMARY

This report outlines the progress so far in our statistical population studies which are designed to provide a firm basis for two relatively new forms of sports drug testing; namely the use of carbon isotope ratio mass spectrometry (CIRMS) for detecting the abuse of endogenous anabolic steroids, and the use of isoelectric focussing to detect the abuse of erythropoietin (EPO). The techniques are very different in methodology and application but both rely on detecting small but significant differences between truly endogenous compounds and their synthetic variants. In order to reliably detect doping with either technique it is essential that the extent of natural variation in the elite athlete population be known. Criteria have been set which are already being used to detect doping but so far no large-scale study has been carried out to test these criteria over a wide range of elite athletes. Our study will provide this information which will not only support the validity of the tests but may also result in improvements in the criteria leading to a more robust and sensitive testing program.

The progress so far is to schedule with over 70% of the samples being analysed for ketosteroids for part 1 of the study (CIR measurements) and almost 70% of the samples being completed for part 2 of the study (EPO glycoforms). The most significant single finding is that the criteria currently in use are entirely appropriate for doping control since no false positives have been found for either method in the hundreds of samples analysed. However preliminary analysis of the CIRMS data has shown that it may be desirable to modify the criteria used to enhance the detection of endogenous anabolic steroids depending on the athletes natural levels. This would be done without compromising the selectivity of the method.

It is anticipated that all goals of the first year of the project will be met on time and within budget. The program for 2004-2005 will result in the completion of part 2 of the study by September 2004 with preparation of a scientific paper on the EPO glycoform study. The second phase of the CIR measurements will begin with analysis of the steroidal diols. During the year a paper will be prepared presenting the data obtained from the completed keto steroid phase of the project.

BACKGROUND

It was recognised in the early stages of the development of an indirect test for detecting recombinant human EPO that it was essential to establish the range of variability expected for all the relevant parameters in a large group of elite athletes. Multiple samples of blood and urine were collected from over 1100 athletes and are currently stored frozen. At the time of the collection it was recognised that the samples represented a valuable resource which could be required to validate future tests for endogenous compounds. Thus the ethics approval obtained from each subject permits the samples to be used in research on the detection of doping with EPO and endogenous steroids. The samples were collected in 12 countries with representation of all major ethnic groups. Because of repeat urine collections there are some 2000 urine samples in total. The factors related to each sample which have been recorded include gender, ethnicity, age, sport, altitude and time since exercise. The sample size and diversity is such that it should be possible to determine if there are any significant effects of gender, ethnicity, exercise, sporting discipline, altitude and biological variation.

The detection and confirmation of the presence of ingested or injected endogenous steroids using CIRMMS is based on the fact that the synthetic versions of the steroids have a lower proportion of carbon 13 resulting in more negative delta ¹³C values compared with naturally produced steroids. Typically synthetic steroids have delta values close to -30 whilst the delta value of natural circulating steroids are typically -22 to -24. The CIRMMS methods used to detect doping measure the delta values of steroid metabolites and compares them to the values found for precursors. The premise on which the method is based is that in a normal individual the delta values of the precursors and the metabolites will be similar. Actual measurement of these values in a wide range of subjects will provide the sound statistical basis on which to base the criteria used for determining a positive doping case.

The current method used to confirm doping with human recombinant EPO is based on the fact that recombinant EPO is significantly less acidic than urinary EPO despite the recombinant product being produced from mammalian cells containing the human gene. This difference in acidity arises from the fact that the recombinant product is less glycosylated and has fewer sialic acid residues than normal urinary EPO. In the normal human body EPO is produced in the kidney in response to low levels of oxygen in the blood. The glycosylation of the EPO protein is needed to allow it to circulate to the bone marrow where it stimulates the production of new red blood cells. The EPO protein is rapidly destroyed and is not effective if injected in the deglycosylated form. The protective effect of the sugar moieties has been extended in the new Amgen product Aranesp, which has the EPO protein modified so that two additional sugar chains are introduced. This has the effect of significantly increasing the half life of the circulating material (Egrie and Browne 2001). Although much is known of the structure of the isoforms of recombinant EPO from mass spectral and other measurements

(Ohta et al 2001) what data there is on the structure of natural EPO comes from a very few subjects. In fact virtually all the data available on the variability of natural EPO isoforms has come from the few IOC laboratories that are currently routinely performing the EPO urine test. Since the urinary EPO test is critically dependent on the difference in glycosylation between natural and recombinant EPO, it is essential to be aware of the extent of natural variability of this glycosylation in the general population of elite athletes.

AIMS

The aims of the project are twofold. Using urine samples already collected from a large cohort of ethnically and geographically diverse elite athletes we intend to:

- A. Determine the variability in the isotope ratio of some selected endogenous steroid metabolites and evaluate whether the delta ^{13}C values are significantly affected by factors such as gender, ethnicity and geographical location.
- B. Determine the variability of the natural isoform pattern of urinary EPO and evaluate whether the patterns are significantly affected by factors such as gender, ethnicity and altitude.

As there are essentially two separate projects within the one grant application the results for each have been reported separately.

Part 1 CIR measurements

Experimental Methods

A method used has been developed for the extraction of the endogenous steroids androsterone (A), etiocholanolone (Et), 11-ketoetiocholanolone (11-keto), and their measurement using CIRMS. After hydrolysis with β -glucuronidase the free steroids are extracted from the urine using BondElut Certify SPE columns. The extracts are analysed by GC-CIRMS using a Finnigan-MAT Delta Plus with a Hewlett-Packard 6890 GC. The calculations of isotopic ratio are made using ISODAT 7.4 software. The $\delta^{13}\text{C}$ values for the steroid metabolites A and Et, and the precursor 11-keto have been measured. The results are stored in Excel spreadsheets prior to statistical analysis.

Results

The results from the first four countries completed Australia, China, Kenya, and New Zealand comprising some 450 samples have been published (Cawley et al 2004). In summary the major findings are:

- The GC-CIRMS methodology has a high degree of precision and accuracy based on multiple measurements of the internal standard 17-MeT. The certified value determined by combustion analysis was -32.80‰, whilst the mean value found was -32.42‰ ($n > 700$), with a SD of 0.90.
- The distribution of $\delta^{13}\text{C}$ values was close to normal in all four countries.
- The $\delta^{13}\text{C}$ values and the spread of these values were similar for the Australian and New Zealand populations. This was to be expected as the dietary patterns in the two countries are similar.
- The $\delta^{13}\text{C}$ values were significantly different ($p < 0.0001$) for China (mean = -21.6), Kenya (mean = -20.3) and Australia/New Zealand (mean = -22.8). It is almost certain that the different dietary patterns are responsible for these differences.
- There was a consistent difference of approximately 2.0‰ between the average $\delta^{13}\text{C}$ values of androsterone and etiocholanolone and the value for the precursor 11-ketoetiocholanolone.
- The most negative $\delta^{13}\text{C}$ value found in any sample for androsterone was -25.1‰ and for etiocholanolone it was -25.8‰.

Implications for Doping Control

These preliminary results have a number significant implications for the use of GC-CIRMS in the detection of the abuse of endogenous anabolic steroids:-

- The precision and accuracy of the multiple measurements adds confidence to the reliability and reproducibility of GC-CIRMS measurements.

Our laboratory like many others use a combination of criteria to assess whether a sample is positive. Our current criteria are

1. The difference between the average of $\delta^{13}\text{C A}$ and $\delta^{13}\text{C Et}$ values, and $\delta^{13}\text{C 11-keto}$ must be greater than 4.0‰.
2. The ratio must be greater than 1.15.
3. $\delta^{13}\text{C A}$ and $\delta^{13}\text{C Et}$ must be more negative than -27.0‰.

All must be met for a sample to be called positive. As expected none of the samples measured so far would be identified as positive.

- The results show that the use of a difference of greater than 4.0‰ is a necessary condition to confirm doping but it is not a sufficient condition. A small number of samples had differences greater than 4.0‰ usually due to isotope enriched (less negative) values for the 11-keto. Thus far a difference of 6.0‰ would be a sufficient condition by itself to confirm doping.
- The use of the ratio of the average of A and Et values to that of the 11-keto provides no additional information and should be discontinued.
- The suggestion has been made that a cutoff value such as -27.0‰ could be used to detect the use of synthetic endogenous steroids (Aguilera et al 2000). This is supported both by the lowest value found being -25.8‰ and by the mean and standard deviation results. The lowest calculated $\delta^{13}\text{C}$ value (mean minus 3σ) is -26.5‰ for New Zealand. The $\delta^{13}\text{C}$ values for Australia, China and Kenya were all less negative.
- The combined criteria of a difference of 4.0‰ and $\delta^{13}\text{C A}$ and $\delta^{13}\text{C Et}$ being more negative than -27.0‰ is appropriate for the Australia/New Zealand populations but will result in a greater number of false negatives when applied to athletes with less negative $\delta^{13}\text{C}$ values such as those from Kenya.
- Although a full statistical analysis has yet to be carried out it is clear from the data so far that the effect of geography is far greater than any other effect including sex and sport. From the nature of human metabolism it would be expected that diet would be the major influence on steroid isotope ratios (Morrison et al 2000).

Part 2 EPO glycoforms

Experimental Methods

The urinary EPO concentrations have been measured using an EPO kit on a DPC Immulite instrument. As this method is designed for serum samples it was necessary to centrifuge each urine sample, ultrafilter it and buffer wash the retentate prior to analysis.

The urinary isoforms were measured using a variation of the original Lasne method (Lasne et al 2002). 20mL of each urine was concentrated to 30uL using two ultrafiltration devices. The concentrated samples underwent isoelectric focussing on an ampholyte gel and were then transferred with two Western blotting steps. The membrane was visualised using Pierce SuperSignal West Femto Maximum Sensitivity Substrate. The signals were recorded with a Fuji LAS-1000 camera and quantitated with Fuji Image Guage v3.41 software. Sample results are stored in an Access database.

Results

In order to properly evaluate the isoform data collected from elite athletes it was first necessary to determine what variability there was in the method itself and what variability was to expected in any one subject over time. The method variability was estimated by analysing urine samples spiked with varying levels of recombinant EPO from 0 to 15 IU/L several times on six gels run on different days. The results showed that a variation of 5 in a value of 50% basic isoforms could be expected (CV = 10%). At higher values (90% basic isoforms) the variation was less being about 3 (CV = 3%). The subject variability was determined by analysing the results from some 20 subjects who had up to 10 urine samples collected over a period of three days. The mean CV observed for the subjects was 17% which is much higher than the variation that could be attributed to the method of measurement. A large variation in urinary EPO concentrations was also observed in these subjects but there was no correlation observed between urinary EPO concentration and % basic isoforms.

Some preliminary results from the EPO2000 samples and the low risk ASDA samples have been evaluated. The table below summarises the results

| | EPO2000 | | ASDA | |
|--------------------|---------|--------|------|--------|
| % Basic Isoforms | Male | Female | Male | Female |
| Mean | 35.8 | 39.7 | 36.5 | 43.9 |
| Median | 35.5 | 37.0 | 35.0 | 44.0 |
| Standard deviation | 14.5 | 16.5 | 13.8 | 15.8 |
| Count | 100 | 50 | 163 | 47 |

The results show that the ASDA low risk routine samples and the EPO2000 samples are not significantly different in the distribution of their EPO % basic isoforms. This demonstrates the validity of including the ASDA samples in our study. From the current data there appear to be no significant effects of ethnicity on the isoform results

| % Basic Isoforms | ASDA males | Chinese males | Kenyan males |
|--------------------|------------|---------------|--------------|
| Mean | 36.5 | 37.2 | 40.3 |
| Median | 35 | 36 | 39 |
| Standard deviation | 13.8 | 17.1 | 11.2 |
| Count | 163 | 38 | 16 |

Implications for Doping Control

These preliminary results have a number significant implications for the use of urinary EPO isoforms in the detection of the abuse of recombinant EPO:-

- The reproducibility of the % basic isoform measurement using the existing amphoteric gel technique with double blotting is adequate for doping control purposes. This was determined under the worst case scenario ie. different gels prepared on different days whereas a positive sample would be measured against standards run on the same gel.
- The standard deviation of the % basic isoforms found for any given individual is approximately 17. This high individual variability means that the detection of differences relating to sport, age or sex is unlikely.
- There have been no differences observed relating to ethnicity.
- The last two findings indicate that the test should be equally applicable to all those tested.
- No positive findings have been found in the samples which indicates that the criteria used to determine doping with recombinant EPO are appropriate. Closer examination of those samples with relatively high % basic isoforms may lead to improved criteria.

Report on Progress

Part 1 CIR measurements

Summary Table

| Activity | Estimated completion in proposal | Progress thus far | On schedule Y/N |
|--------------------|-------------------------------------|--|-----------------|
| Organise logistics | July 2003 | Staff were allocated to the project in July 2003 | Y |
| Organise samples | August 2003 | Samples selected from existing sample set and sorted by country of origin and volume of sample available. ¹ | Y |
| Analyse samples | June 2004 | Approximately 900 samples have been extracted for GC-CIRMS analysis. 750 have been analysed. | Y |
| Evaluation of data | Nov/Dec 2003 and April to June 2004 | The data evaluation needed for the results entry into the database has been completed for some 700 samples from 12 countries. The statistical evaluation will begin in April 2004. | Y |
| Report to ADRP | January 2004 | Report requested in November 2003. Preliminary report submitted in December. Second report requested in March 2004. | Y |

1. The original proposal called for the analysis of most of the 2000 urine samples collected in the EPO2000 project. As there were only a little over 1100 subjects in the study many of the urine samples were duplicate collections made a week or so apart. Close examination of the samples collected has shown that not all are suitable for analysis. In addition it has been found that steroid excretion is normally stable for an individual over extended periods of time and repeat analyses from the same subject will provide virtually no additional information. As a result of these factors the number of urine samples suitable for analysis by GC-CIRMS is approximately 1050.

Part 2 EPO glycoforms

Summary Table

| Activity | Estimated completion in proposal | Progress thus far | On schedule Y/N |
|--------------------|----------------------------------|--|-----------------|
| Organise logistics | July 2003 | Staff were allocated to the project in July 2003 | Y |
| Organise samples | August 2003 | Samples selected from existing sample set and criteria established for selection of further samples. ¹ | Y |
| Analyse samples | June 2004 | Urinary EPO concentrations have been measured for over 1100 EPO2000 samples. Only one quarter of the urines had sufficient EPO present to continue with isoform analysis. The data set has been extended by using samples from the operational sample base. Over 600 of these have been screened for urinary EPO concentration and some 300 have had isoform distributions measured. | Y |
| Evaluation of data | January and July 2004 | Isoform measurements have been made but statistical evaluation is currently behind schedule. | N |
| Report to ADRP | January 2004 | Report requested in November 2003. Preliminary report submitted in December. Second report requested in March 2004. | Y |

1. In the original project proposal it was planned to use the EPO2000 urine samples to obtain the data required. However only approximately one quarter of the samples had sufficient urinary EPO present to proceed with EPO isoform analysis. This meant that there would be significantly less data available than expected. It is possible that these samples, despite being stored frozen, have undergone some degradation. Since the project was planned there has been evidence coming from several laboratories that some urine samples can change with time and produce isoform patterns that resemble recombinant EPO (Howe 2003). With fresh urines 40 to 50% have sufficient EPO to proceed with isoform analysis. For these reasons it was decided to continue the project using samples taken from the routine operational collections. Samples which have been reported negative are selected from a range of sports including some at risk endurance sports but avoiding samples where there is any indication at all from blood parameters of EPO abuse. In addition fresh urine samples have been obtained from elite athletes in Indonesia to extend the ethnic diversity and it is planned to obtain urine samples from at least one other country.

Proposed activities to June 2004

For the period up to June 30 it is anticipated that the project will continue in accordance with the schedule presented in the original project proposal. Both projects are on schedule or ahead of schedule for experimental activities. The specific activities proposed for the next months are:

- **Part 1 CIR measurements.** Extractions and GC-CIRMS analyses will continue with the intention of having approximately 1050 samples from 12 countries analysed by June 2004. Data processing and review will continue so that the statistical analysis can begin. The method for the diol analysis procedure will be finalised and validated in preparation for the second stage of the project. It appears at this stage that the method will be considerably more complex than first envisaged and the number of urine samples to be analysed will need to be reduced.
- **Part 2 EPO glycoforms** Analysis will continue so that 600 urine samples will have been tested using the gel electrophoresis method. It is anticipated that at least 25% of the samples will be from subjects having non-Caucasian ethnicity. The statistical analysis of the data will commence before June 2004 but will not be completed until September as indicated in the original proposal.

EXPENDITURE

The funds expended for the period from July 2003 to the end of February 2004 are set out in the table below:

| Expense Category | Amount Approved | Amount Spent |
|------------------------------|-----------------|--------------|
| Salary, scientific personnel | \$50,000 | \$21,000* |
| Salary, technical personnel | \$61,000 | \$30,000 |
| Consumables | \$120,628 | \$99,000 |
| Overheads | \$30,500 | \$27,000 |
| Totals | \$262,128 | \$177,000 |

* There have been major savings in the professional staff salaries this year because the person carrying out the CIR aspect of the project is now a full time Ph.D. student with a scholarship provided by the University of Sydney. Major aspects of the ketosteroid CIR work form part of his research.

Proposal for Year 2

The original proposal was for a two-year program with the major expenditure occurring in the first year. In the second year the EPO glycoform project will be completed within the first four months and will involve statistical of the data and preparation of a scientific paper summarising the work and its major findings. The CIR measurements will continue but the methodology used will change. The ketosteroid analysis will be essentially complete and the analysis of steroidal diols will begin. The ketosteroid analysis is much simpler than the diol analysis but in some cases of doping with endogenous steroids the diol methodology is the only one that will give a definitive result. We had hoped to develop a simple method for diol extraction and purification prior to GC-CIRMS analysis such as we have done for the ketosteroids, but it appears at this stage that an extensive cleanup using HPLC will be required. Because of this the number of samples that can be analysed using the resources available will need to be reduced from the 1000 stated in the original budget. On current estimates the number of samples that can be analysed will be approximately 500. The samples to be analysed will be chosen from the EPO2000 samples using the data already obtained from the ketosteroid analyses with a view to optimising the subject diversity and hence the statistical significance of the data obtained.

The major objectives for Year 2 are –

- Complete the statistical analysis of the EPO glycoform results and prepare a publication on the results of this study.
- Complete the statistical analysis of the ketosteroid GC-CIRMS measurements and prepare a publication on the results of this study.
- Begin the analysis of steroidal diols using GC-CIRMS with the view to having a statistically significant dataset completed by mid 2005.

References

- Aguilera, R., Chapman, T.E., Catlin D.H., (2000) A rapid screening assay for measuring urinary androsterone and etiocholanolone delta ^{13}C values by gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry*, 14:2294-2299.
- Cawley A., Rogerson J., Rahman K., Trout G., Kazlauskas R. (2004) Preliminary results on the carbon isotope ratios of ketonic steroids in urine samples collected from different countries. *Recent Advances in Doping Analysis* (11). W. Schanzer, H. Geyer, A. Gotzmann, and U. Mareck-Engelke (eds), Sport und Buch Strauss, Cologne, 2004, 183-193.
- Egrie, J.C., Browne J.K., (2001), Development and characterisation of novel erythropoiesis stimulating protein (NESP). *Brit J. Cancer* 84(Suppl 1):3-10.
- Howe C. (2003) Personal communication from F. Lasne of LNDD Paris
- Lasne F, Martin L., Crepin N., de Ceaurriz J. (2002) Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones, *Anal. Biochem.*, 311, 119-126.
- Morrison, D.J., Dodson, B., Slater, C., Preston, T. (2000) Carbon 13 natural abundance in the British diet: implications for ^{13}C breath tests. *Rapid Communications in Mass Spectrometry*, 14:1321-1324.
- Ohta, M., Kawasaki, N., Hyuga, S., Hyuga, M., Hayakawa, T., (2001) Selective glycopeptide mapping of erythropoietin by on-line high-performance liquid chromatography-electrospray ionization mass spectrometry.
- Shackleton, C.H., Phillips, A., Chang, T., Li, Y., (1997), Confirming testosterone administration by isotope ratio mass spectrometric analysis of urinary androstenediols. *Steroids*, 62:379-387.



Analysis of quantization error in high-precision continuous-flow isotope ratio mass spectrometry

Gavin L. Sacks, Christopher J. Wolyniak, J. Thomas Brenna*

Savage Hall, Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853, USA

Received 10 June 2003; received in revised form 8 August 2003; accepted 12 August 2003

Abstract

High-precision isotope ratio mass spectrometry (IRMS) systems are equipped with digitizers that deliver effective maximum digitization depths of 16 to 24 bits; however, there are no analyses of the proper board depth required to retain high precision in continuous-flow techniques. We report an experimental and theoretical evaluation of quantization error in continuous-flow IRMS (CF-IRMS). CO₂ samples (100 pmol–30 nmol) were injected into a gas chromatography combustion IRMS system (GC-CIRMS). The analog signal was digitized by high precision, 24-bit ADC boards at 10 Hz, and was post-processed to simulate 12, 14, and 16-bit data sets. $\delta^{13}\text{C}_{\text{pdb}}$ values were calculated for all data sets by the conventional “summation” method or by curve-fitting the chromatographic peaks to the exponentially modified Gaussian (EMG) function. Benchmarks of S.D.($\delta^{13}\text{C}_{\text{pdb}}$) = 0.3, 0.6, and 1.0‰ were considered to assess precision. In the presence of significant quantization noise, curve-fitting required several-fold less CO₂ than the summation method to reach a given benchmark. We derived an equation to describe the theoretical limitations of precision for the summation method as a function of CO₂ admitted to the source and the step size of the boards. Theory was in close agreement with the observed lower limit of precision for the simulated 16-bit data set. Curve-fitting achieved a precision of S.D. <0.3‰ for injections 20-fold smaller than summation for CO₂ samples collected on an IRMS with 16-bit resolution. By mitigating the impact of quantization noise, curve-fitting expands the dynamic range within a single run to include lower analyte levels, and effectively reduces the need for high pumping capacities and high precision ADC boards.

© 2003 Elsevier B.V. All rights reserved.

Keywords: Isotope ratio mass spectrometry; Mass spectrometry; Quantization error; Carbon dioxide

1. Introduction

Isotope ratio mass spectrometry (IRMS) coupled to a gas chromatography–combustion interface (GC-C) can routinely measure relative differences in $^{13}\text{C}/^{12}\text{C}$ isotope ratios to a precision of few parts per million for samples containing 10 ng of sample or less

[1,2]. GC-CIRMS data consists of three concurrent chromatographic traces ($^{44}\text{CO}_2$, $^{45}\text{CO}_2$, $^{46}\text{CO}_2$) from three detectors operated in parallel. Achieving high precision requires careful and consistent definition of background levels and peak integration for all three traces. Most commonly, peak areas are integrated by the “summation” method. The start and end of a peak are detected, and the background is described as a square or trapezoidal area beneath the peak. Raw data are summed over the length of the peak, and the background area is subtracted. Ricci et al. [3] described two

* Corresponding author. Tel.: +1-607-255-9128; fax: +1-607-255-1033.

E-mail address: jtb4@cornell.edu (J.T. Brenna).

general methods for determining the background using summation; the “individual summation” method, in which the background is defined by connecting low points on either side of the peak, and the “dynamic summation” method, in which low points are connected throughout the chromatogram regardless of the location of peaks.

All IRMS instruments use digitizers to convert analog signal from Faraday cups to digital data, which must be processed to yield isotope ratios. The precision of a digitizer is expressed in terms of bits, where an N -bit board has 2^N steps over a given range. As an example, a 16-bit board has ~65 000 steps; if the board has a range of 0–10 V, then the step size of the board is ~0.15 mV. The rounding of a continuous signal to discrete steps introduces noise, which is referred to as quantization error or “bit noise”. This effect is shown graphically in Fig. 1, where simulated Gaussian peaks of 24, 16, 14, and 12-bit resolution are presented. At high resolution (24 bits), no quantization noise is noticeable, and the peak appears as a smooth trace. As the resolution decreases, steps become obvious, and the shape of the peak deteriorates. The quality of data reduction in continuous-flow IRMS must depend at least in part on the digitizer depth because the intensity level established for peak start and stop depends on this parameter. As depth decreases, the intensity of

the background is, in general, less well represented by the intensity levels of the peak’s start and stop points. There are no analyses available that establish the relationship between isotope ratio precision and digitization depth.

The effect of digitization depth on precision and accuracy is inextricably linked to data reduction algorithms.

The reproducibility of the summation background correction depends in part on the two points that anchor the background line under the peak; imprecision in the measurement of either point multiplies through the entire length of the background segment connecting the points. In the presence of a simple linear background, a background line is easily drawn between any two points on either side of the peak, as shown in Fig. 2a. Chemical noise due to column bleed or contaminant peaks may cause inaccuracy in defining the background, but such noise is usually correlated in all three traces. This covariance may mitigate the effects of chemical noise on the calculated isotope ratio. However, in the case of quantization error, as shown in Fig. 2b, the magnitude and direction of error is uncorrelated among the three traces, and this then poses a special case. Our previous work has shown that peak integration by curve-fitting improves precision and accuracy in cases of low signal-to-noise [4] and overlapping peaks [5]. Background correction

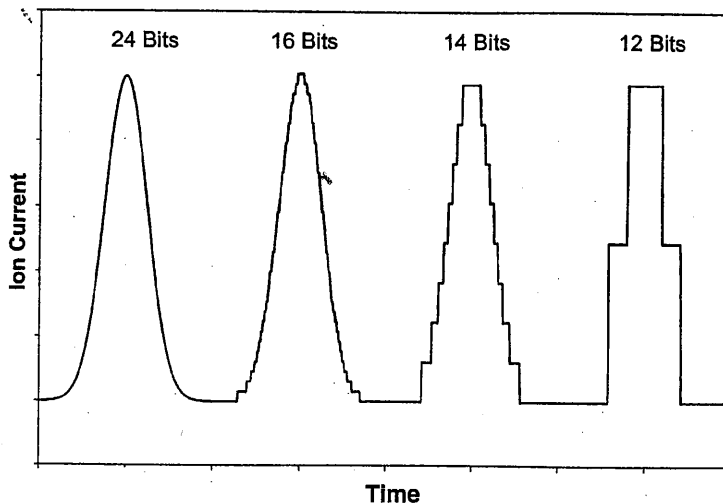


Fig. 1. A simulation of a Gaussian signal collected by ADCs of various resolutions (24, 16, 14, and 12 bits) and quantization errors. At 24-bit resolution, quantization error is not visible, and the peak appears as a smooth trace. At 16 bits, bit noise is evident primarily at the base of the peak. At 12-bit resolution, the signal is barely recognizable as a Gaussian shape.

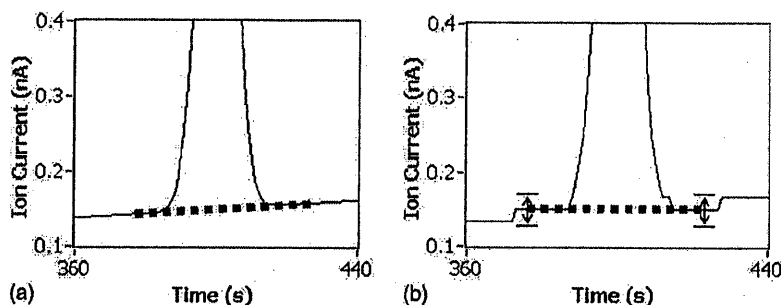


Fig. 2. Simulated chromatographic peaks in the presence of a linearly rising background (a) without and (b) with quantization error. In the presence of quantization error, the true background may fall anywhere within the arrows. Without quantization error background is easily and accurately achieved by connecting points on either side of the peak.

in curve-fitting is not constrained to the actual values represented by the discrete digitization levels, and we hypothesized that it may not be as sensitive to quantization error as summation.

Quantization error is typically not dominant in GC–CIRMS when high precision IRMS data acquisition systems use sufficiently deep digitization boards and signals are sufficiently strong. Noise from other sources, such as chemical noise, is greater than the step size of the digitizers. However, quantization error may become important in two specific situations: (a) in data reduction of minor peaks in a chromatogram where there are fewer steps between baseline and peak top, and (b) when low precision digitizers are used, as is common in low cost IRMS instruments designed primarily for measurements of high abundance samples, such as CO_2 in breath tests. In addition, these systems usually have lower pumping capacity, which limits the flow rate that the IRMS source accepts. The lower inlet flow rates result in smaller signals for equivalent analyte abundance via higher split ratios, making quantization error more prominent. In this report, we evaluate quantization error theoretically and experimentally to determine the limiting the precision achieved by the conventional summation algorithm and by curve-fitting.

2. Experimental

2.1. Instrumentation

A Varian 3400 GC system was coupled via a combustion furnace to one of two gas IRMS instruments:

(a) a FinniganMAT 252 (FMAT252) run in high linearity mode, or (b) an Analytical Precision Products 2003 (APP2003). Both IRMS systems were operated with a source pressure of 1×10^{-6} Torr and had an absolute sensitivity of ~ 5000 mol/ion (1 Torr = 133.322 Pa). The GC–C system is described in detail elsewhere [6]. Briefly, the effluent from the capillary column (60 m \times 0.32 mm, 0.25 μm , BPX70; SGE, Austin, TX, USA) is directed to a combustion furnace filled with CuO and held at 850 $^\circ\text{C}$, and dried in a Nafion water trap before admittance to the IRMS system through an open split. Since CO_2 gas was injected as a sample, the combustion step was not necessary, but was retained in the system to increase the verisimilitude to real GC–CIRMS operating conditions. The FMAT252 has differential pumping and a higher overall pumping capacity, while the APP2003 has only a single turbopump. As a result, the FMAT252 can tolerate higher inlet flow rates. The open split of the FMAT 252 accepted 0.2 ml/min (split ratio = 8.4:1), and the open split of the APP2003 accepted 0.07 ml/min (split ratio = 24:1).

CO_2 (Airgas East, 99.9%) injections were performed by hand consecutively. The split ratio and the injection size were varied to yield between 100 pmol and 30 nmol on column. Four or five replicates were performed for each injection size. The moles of CO_2 in each injection were approximated by assuming ideal gas conditions.

Data was collected on the FMAT 252 using SAXI-CAB [7], a laboratory-built LabVIEW-based [8] data acquisition system employing National Instruments (Austin, TX, USA) 435 \times digitizers yielding

24 bits operating at 10 Hz. Data were collected on the APP2003 using the vendor-supplied 16-bit, 10 Hz data acquisition system. Both systems simultaneously monitored the $m/z = 44$, 45, and 46 cups with >99% duty cycle.

2.2. Data processing

Before data reduction, data collected from the FMAT 252 at 24 bits was rounded on all three traces to simulate 16-, 14-, and 12-bit data sets. The head amplifiers have a maximum signal of 10 V (33 nA for $m/z = 44$), so the step size, Δ , for a given board depth was calculated as:

$$\Delta = \frac{10 \text{ V}}{2^{\text{bits}}} \quad (1)$$

We created simulated data sets by rounding data points to the nearest step:

$$\text{data(quantized)} = \text{round} \left[\frac{\text{data(raw)}}{\Delta} \right] \Delta \quad (2)$$

where, the *round* function rounds the input to the nearest whole number.

The 16-bit data from the APP2003 was used without modification. All data sets were processed using SAXICAB by either the individual summation method or by curve-fitting. The individual summation method used by SAXICAB was adapted from Ricci et al. [3]. Starts and stops of peaks were determined with a slope sensitivity of 0.3 nA/s. The lowest point 2 s before and 2 s after the peak limits were located, and a straight line was drawn between the two points to define the background. In the curve-fitting algorithm, the traces were fit to exponentially modified Gaussian (EMG) functions using the Levenberg–Marquardt algorithm. Mathematical details of the EMG function can be found elsewhere [9].

High-precision isotope ratios are expressed in the delta (‰) notation:

$$\delta^{13}\text{C}_{\text{pdb}} = \frac{{}^{13}\text{R}_{\text{spl}} - {}^{13}\text{R}_{\text{pdb}}}{{}^{13}\text{R}_{\text{pdb}}} \times 1000 \quad (3)$$

where ${}^{13}\text{R}_x$ is the ratio of ${}^{13}\text{C}$ to ${}^{12}\text{C}$, SPL refers to the sample, and PDB refers to the international standard, Pee Dee Belemnite, where ${}^{13}\text{R}_{\text{pdb}} = 0.0112372$. In our work, $\delta^{13}\text{C}$ of the CO_2 injections were calculated using pulses of standard CO_2 gas that had been indirectly

calibrated to the PDB reference. The contribution of ${}^{17}\text{O}$ to the ${}^{45}\text{CO}_2$ signal was taken into account by the method of Santrock et al. [10]. No outliers were excluded from the reported data.

3. Results and discussion

3.1. Observed effects of quantization error

Fifteen CO_2 injection amounts were used to produce peak areas on the FMAT 252 that varied over 2.5 orders of magnitude. The peaks showed excellent symmetry and narrow peak widths, with a full width at half maximum of <3 s. The mean reproducibility of the area for each injection size, as measured by the area of the $m/z = 44$ signal, was R.S.D. = 13%. Plots of $\delta^{13}\text{C}_{\text{pdb}}$ versus injection size, shown in Fig. 3, are displayed for both the curve-fitting (a) and individual summation (b) methods. The plots are similar in appearance to those presented by others [11] investigating the performance of GC–CIRMS at low signal levels.

In agreement with our previous work [4], we observe modest improvement of precision at low signal levels using the curve-fitting method. Using the summation method, precision deteriorates (S.D. > 1.0‰) for injection sizes less than 400 pmol on column (~50 pmol to the IRMS). Curve-fitting improves this limit to 175 pmol on column (~20 pmol to the IRMS). The integration methods performed comparably and acceptably at large injection sizes. For on-column injections of at least 6.8 nmol (~800 pmol to the IRMS), the individual summation method had a precision of S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) = 0.1‰. The curve-fitting method gave slightly worse precision for large injections, S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) = 0.2‰. It is not obvious why the summation method out-performs the curve-fitting method for very large sample sizes. One possibility is that the precision of the curve-fitting method is limited by differences between the shape of the model EMG function and the shape of real, chromatographic peaks. In this case, increasing the injection size past a certain point would not improve the fit, even though S/N is increasing. Using a different function to describe the peaks may further improve results.

To assess the tolerance of the two integration methods to quantization noise, we evaluated simulated

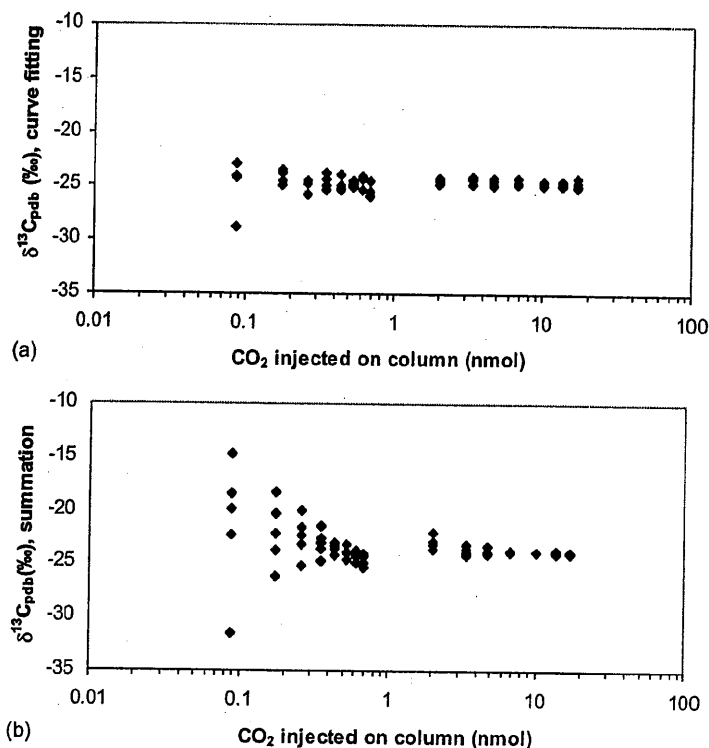


Fig. 3. $\delta^{13}\text{C}_{\text{pdb}}$ vs. CO_2 injected on column for (a) curve-fitting and (b) individual summation algorithms. Data was collected using a homebuilt system at 24 bits from FMAT252.

12, 14, and 16-bit raw data sets generated from the raw 24-bit FMAT252 data, and processed both by curve-fitting and summation. The accuracy of successive injections was very good, even in the presence of bit noise. For each method, the mean $\delta^{13}\text{C}$ for any two injection sizes did not differ significantly. Plots of $\text{S.D.}(\delta^{13}\text{C}_{\text{pdb}})$ versus CO_2 injected on column at all bit resolutions are shown in Fig. 4. Each plot appears to extend asymptotically along the x - and y -axes, and we can evaluate the dependence of precision on quantization error visually; poor performance is indicated by the asymptotic plot moving up and away from the axes. At 24-bit resolution, plots of the summation and curve-fitting methods nearly overlap, except at very small injection amounts, indicating that performance is similar. With increasing quantization error, the minimum amount of CO_2 necessary to reach a given level of precision increases rapidly for the summation method. Curve-fitting is more forgiving; precision from 14- and 16-bit data is comparable to

the 24-bit data. The plot for 12-bit resolution shows some loss of precision, but does not fare as badly as the summation method.

To evaluate the methods objectively, we defined $\text{S.D.}(\delta^{13}\text{C}_{\text{pdb}}) = 0.3, 0.6$ and 1.0% as benchmarks for high precision. The data were least-squares fitted to a power function, of the form:

$$\text{S.D.} = A[\text{CO}_2]^B \quad (4)$$

where $[\text{CO}_2]$ is the moles of CO_2 injected on column, S.D. is the observed precision, and A and B are constants. The power function was chosen for empirical reasons, because it modeled the observed data acceptably, and the fitted curves can then be compared. The best-fit lines for both the summation (dashed) and curve-fitting (solid) methods are shown in Fig. 4. From the best-fit equations, we calculated the amount of CO_2 injected on-column necessary to achieve the 0.3, 0.6, and 1.0% benchmarks (Fig. 5). With least quantization error (24-bit resolution), the

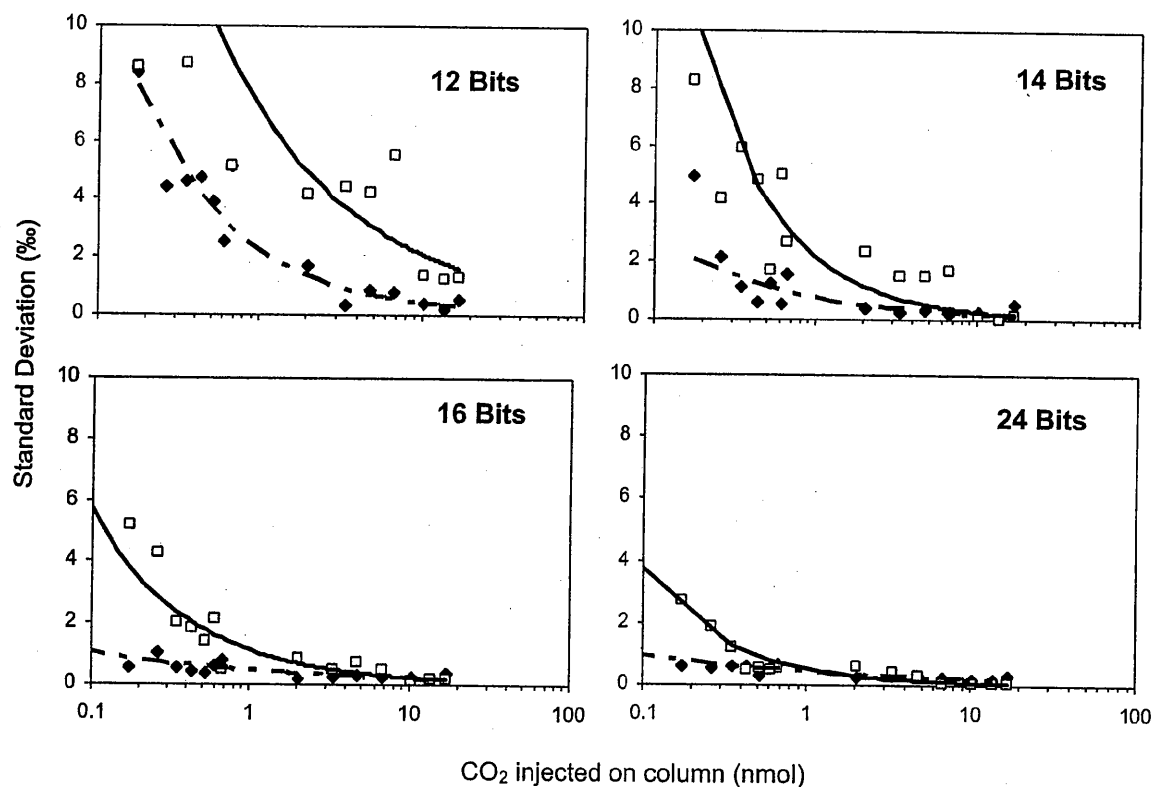


Fig. 4. S.D.($\delta^{13}\text{C}_{\text{pdb}}$) vs. CO₂ injected on column at 12-, 14-, 16-, and 24-bit resolutions, calculated by summation (\square) or curve-fitting (\blacklozenge) algorithms. Each point represents four or five replicates. The data for each method and each resolution was fit to a power equation (general form: $\text{S.D.} = A[\text{CO}_2]^B$, and the best-fit lines are drawn for both methods (solid line: summation; dashed line: curve-fitting).

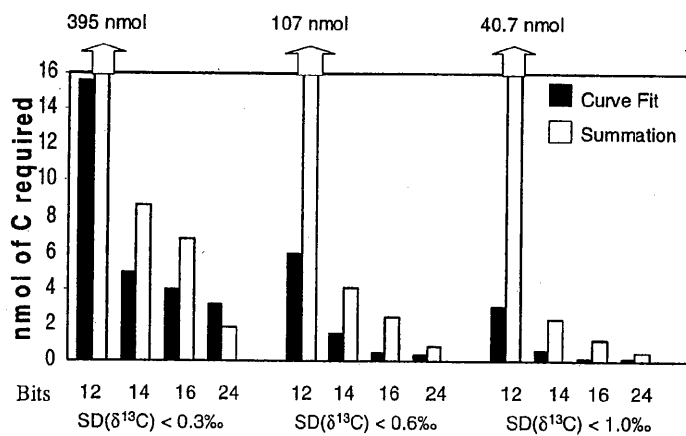


Fig. 5. Carbon required on-column, in nanomoles, to reach a specified level of precision for a given ADC board resolution. Results are shown for data reduced by curve-fitting and summation algorithms.

summation method requires slightly less CO₂ than the curve-fitting method at the 0.3‰ benchmark (1.86 nmol versus 3.19 nmol). At 16-bit resolution, the summation method requires 6.83 nmol, an increase of 267%, compared to a 25% increase for curve-fitting over the same interval. To reach the 0.6‰ benchmark at 16 bits, summation requires an increase of 224% (from 0.77 to 2.50 nmol), compared to 33% for curve-fitting. At 12-bit resolution, the amount of CO₂ on column necessary to achieve S.D. = 0.6‰ by summation is 107 nmol, which far exceeds the capacity of the GC column. Curve-fitting requires only 6 nmol to reach S.D. = 0.6‰ at 12-bit resolution. As was discussed previously, curve-fitting is superior to summation at the 1.0‰ benchmark, even in the absence of quantization noise. At 24 bits, curve-fitting requires 80 pmol to achieve S.D. = 1.0‰, five-fold less than summation; a similar level of improvement in precision is seen at 14- and 16-bit resolution.

To summarize, in the absence of quantization noise, similar amounts of CO₂ are necessary to achieve precision of 0.3–0.6‰ for both integration methods. The summation method requires a dramatic increase in the injection size to maintain this level of precision in the presence of quantization noise, while the curve-fitting method is relatively unaffected. At a lower standard of precision (S.D. = 1.0‰), curve-fitting is superior regardless of the magnitude of quantization error.

3.2. Theoretical limits of quantization error on precision

In IRMS, the signal is recorded as a voltage proportional to the ion current, and can be reported in amperes or in volts. If the signal is reported in volts, the area of the $m/z = 44$ signal, A_{44} , is related to the moles of ⁴⁴CO₂ that enters the IRMS, [⁴⁴CO₂], by the equation:

$$A_{44} = [\text{}^{44}\text{CO}_2] \frac{N_a e}{E} R_\Omega \quad (5)$$

where N_a is Avogadro's number, e is the fundamental charge, E is the absolute sensitivity of the IRMS in molecules/ion, and R_Ω is the feedback resistance of the amplifier.

In the summation method, the background is defined by drawing a line between two background points, (t_1, y_1) and (t_2, y_2) ; the background area, A , is the

trapezoidal region between this line and the time axis. We can calculate this area by the equation:

$$A(\text{background}) = \frac{1}{2} W(y_1 + y_2), \quad (6)$$

where $W = t_2 - t_1$

Quantization noise is uniformly distributed over an interval and the error for a single measurement is:

$$\sigma_y = \frac{\Delta}{\sqrt{12}} \quad (7)$$

where Δ is the minimum step size of the acquisition boards. A full derivation of this can be found in Haykin's text on digital communication [12]. Assuming that quantization error at y_1 and y_2 is uncorrelated, we can use standard techniques for propagation of errors to determine the total quantization error in measuring the background area, σ_A :

$$\sigma_A = \frac{W\Delta}{2\sqrt{6}} \quad (8)$$

It has been noted that evaluating the effect of chemical noise on precision of isotope ratios is difficult, because this noise is usually highly correlated between the major and minor traces [11]. Unlike chemical noise, quantization noise on each trace should be uncorrelated. This greatly simplifies calculation of the propagation of errors for the relation of the observed isotope ratio, ⁴⁵ R_{obs} , to the actual isotope ratio, ⁴⁵ R_{act} :

$${}^{45}R_{\text{obs}} = \frac{A_{45} \pm \sigma_{45}}{A_{44} \pm \sigma_{44}} = {}^{45}R_{\text{act}} \pm \sigma_{\text{obs}} \quad (9)$$

where

$$\sigma_{\text{obs}} = {}^{45}R_{\text{act}} \sqrt{\left(\frac{\sigma_{45}}{A_{45}}\right)^2 + \left(\frac{\sigma_{44}}{A_{44}}\right)^2} \quad (10)$$

The standard error can be rearranged and expressed in terms of parts per thousand:

$$\begin{aligned} \sigma_{\text{ppt}} &= 1000 \times \frac{\sigma_{\text{obs}}}{{}^{45}R_{\text{act}}} \\ &= 1000 \times \sqrt{\left(\frac{\sigma_{45}}{A_{45}}\right)^2 + \left(\frac{\sigma_{44}}{A_{44}}\right)^2} \end{aligned} \quad (11)$$

At natural abundance, $A_{45} \sim 0.011A_{44}$. Assuming the feedback resistance is 100× larger for the 45 cup than for the 44 cup, $\Delta_{45} = 0.01\Delta_{44}$. Combining this and Eqs. (5), (8) and (11), we arrive at:

$$\sigma_{\text{ppt}} = \frac{276W\Delta_{44}E}{[\text{}^{44}\text{CO}_2]N_a e R_\Omega} \quad (12)$$

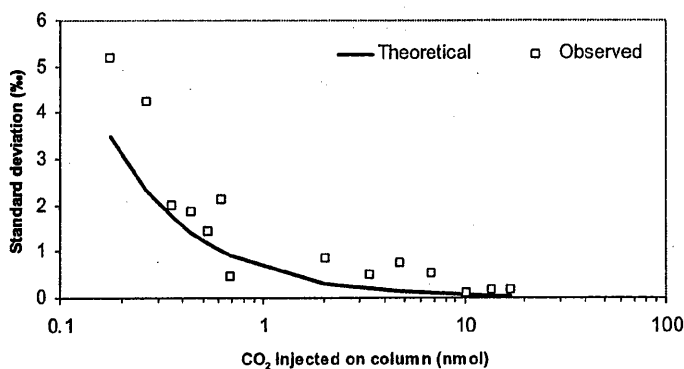


Fig. 6. S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) vs. CO_2 injected on column for simulated 16-bit data. The line indicates the theoretical limit of precision as a function of injection size, as calculated from Eq. (12).

At natural abundance, a standard deviation of $\sigma_{\text{ppt}} = 1.0$ is approximately equivalent to $\text{S.D.}(\delta^{13}\text{C}_{\text{pdb}}) = 1.0\%$. We used Eq. (12) to predict the standard deviation as a function of injection size at 16-bit resolution, compensating for an open split ratio of 8.4:1. The integration window, W , was assumed to be constant at 10 s, $E = 5000$, and $R_{\Omega} = 3 \times 10^8 \Omega$. A plot of the calculated limits compared to the observed precision at 16 bits is shown in Fig. 6. There is good agreement between theory and experiment. The calculated precision is within a factor of five of the observed precision for all injection sizes. More striking, the calculated precision is a “lower limit”, as nearly all the measured precisions lie above the theoretical prediction. The biggest discrepancies occur for large injection sizes, where the effect of quantization error is minimized, and other sources of error (e.g. contaminants) may dominate.

Eq. (12) can also be used to demonstrate that quantization error should be negligible for signals acquired with 24-bit digitizers. Eq. (12) predicts that only 0.6 pmol of CO_2 to the IRMS should be necessary to achieve a precision of 0.5% if quantization error is the only limiting factor. However, counting statistics dictate that a minimum number of ions must be formed to achieve a specified precision to overcome the shot-noise limit. Merritt and Hayes [11] give this equation as:

$$\sigma_{\delta}^2 = \frac{(2 \times 10^6)(1 + R)^2}{EmN_a R} \quad (13)$$

where σ_{δ} is the shot noise limited standard deviation, R is the natural abundance isotope ratio, E is the ionization efficiency, m is the moles of CO_2 , and N_a is Avogadro's number. Substituting $E = 5000$ and $R = 0.011$, we find that 6 pmol of analyte is required to achieve $\text{S.D.} < 0.5\%$. Therefore, when high precision 24-bit boards are used, the effect of quantization error is superseded by shot noise.

Our theoretical treatment gives insight into why curve-fitting is less sensitive to quantization error than summation. In the summation methods we have discussed, imprecision in a single data point chosen as the background is multiplied throughout the background correction; for a peak width of N data points, the total quantization error scales as N . In contrast, the algorithms used in curve-fitting minimize the sum of squares between the fit curve and every data point. In curve-fitting, the quantization noise for each individual point is averaged over the entire curve; for N data points, the total quantization error scales as $N^{1/2}$. For a peak width of 10 s and a sampling rate of 10 Hz, this translates into a 10-fold reduction of quantization error.

The theoretical treatment we describe is appropriate for understanding the effects of quantization error on summation integration methods that choose single points on either side of the peak to define a background. It does not examine the limits of other classes of data reduction methods. An obvious improvement to the summation integration method would be to average n points on either side of the peak, which would increase the effective number of bits of

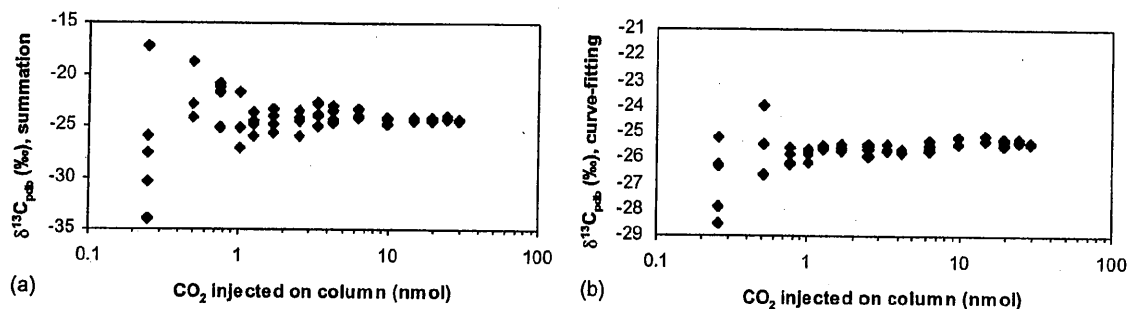


Fig. 7. $\delta^{13}\text{C}_{\text{pdb}}$ vs. CO_2 injected on column for (a) individual summation and (b) curve-fitting algorithms. Data was collected at 16 bits and 10 Hz on the APP2003.

the background measurement by $n^{1/2}$. While this approach could work well for isothermal runs with constant background, it is much less suitable for complex GC–CIRMS chromatograms, where it is not obvious which points should be averaged; that is, which points represent pure background and do not contain chemical noise or the tail ends of peaks. Ricci et al., observed that the averaging method gives slightly higher background values than other corrections [3]. They also reported that the dynamic background correction (which uses single points) yielded improved δ -values over the averaging method. Thus, a method may be insensitive to quantization error, but may still give worse results due to other variables.

3.3. Improving precision on a 16-bit IRMS

To test the effectiveness of curve-fitting on GC–CIRMS data acquired by low precision digitiz-

ers, we ran multiple CO_2 injections on an APP2003 using 16-bit digitizers, and otherwise in similar fashion to the work on the FMAT252. A plot of $\delta^{13}\text{C}_{\text{pdb}}$ versus injection size is shown for summation (Fig. 7a) and curve-fitting (Fig. 7b). Fig. 8 shows a plot of S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) as a function of injection size for both integration methods. The theoretical limit on the summation method, calculated from Eq. (12), is shown in the same figure as a dashed line. The observed precision for the summation method agrees well with theoretical predictions; most of the data points lie just above the lower limit curve. Almost 15 nmol of CO_2 on column are necessary to achieve a precision of $<0.3\%$ using the summation method. Using curve-fitting, only 0.76 nmol are necessary to reach that level of precision, a 20-fold improvement. This is greater than the two-fold advantage seen by curve-fitting the 16-bit data from the FMAT 252. One possible explanation is that the APP2003 data is

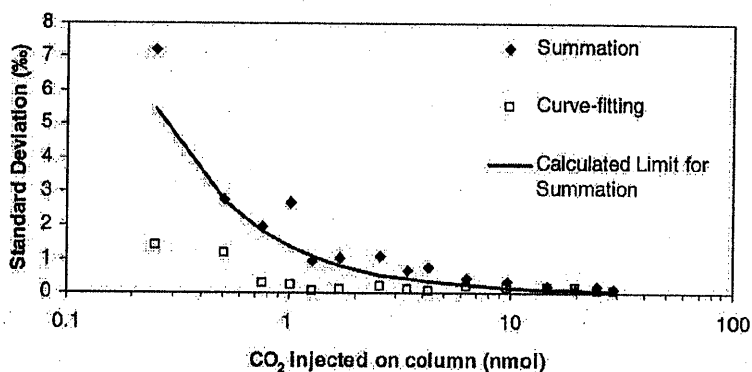


Fig. 8. S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) vs. CO_2 injected on column for runs on APP2003 with 16-bit boards. The dashed line indicates the theoretical limit of quantization error on precision as a function of injection size, as calculated from Eq. (12).

affected primarily by bit noise, while the FMAT252 data has other sources of noise that cannot be eliminated by curve-fitting. The FMAT252 accepts a three-fold higher flow rate than the APP2003, so there is likely more chemical noise in the FMAT252 signal.

The relative immunity to quantization error with curve-fitting permits the IRMS to be run at lower inlet flow rates, which effectively increases quantization noise by decreasing the number of steps between background and peak with relatively little influence on chemical noise. The advantages of lower inlet flow rates are a longer lifetime for the filament, and reduced need for pumps and pumping capacity. These benefits, plus the reduced need for expensive ADC boards, should make high-precision GC–CIRMS more amenable to portable and low-cost applications. In principle, statistical considerations define the lower limits of flow rates. However, counting statistics dictate that S.D. = 0.5% requires 6 pmol of CO₂ to the source for a typical continuous flow IRMS ($E = 5000$), and GC–CIRMS applications usually work well above this limit. Thus, modestly lower resolution and inlet flow rates should not significantly affect performance, so long as appropriate integration techniques are used.

4. Conclusions

Data reduction using curve-fitting is more robust than the conventional summation method in the presence of even modest levels of quantization error. Using data obtained on high precision digitizers, the curve-fitting algorithm required several-fold less CO₂ to reach benchmarks of high precision (S.D. = 0.3, 0.6, and 1.0%) at any of the three simulated board depths (12, 14, or 16 bits). The poor performance of the summation algorithm was particularly noticeable at the 12-bit resolution, where S.D. < 1.0% could not be reached even at the maximum injection size allowed by the dynamic range of the Faraday cups. We

have derived an expression that describes the influence of quantization noise on isotope ratios calculated from raw IRMS data, and shown that it accurately predicts the lower limit of precision. Our theoretical treatment assumes that quantization error is uncorrelated between the $m/z = 44$ and 45 signals, and is appropriate for any data reduction algorithm that uses single points on either side of the peak to describe the background.

Curve-fitting substantially improved precision on GC–CIRMS data collected by an instrument with 16-bit digitizers. The summation algorithm required 15 nmol of CO₂ on-column to achieve a precision of S.D. = 0.3%, while curve-fitting required only 0.76 nmol. Thus, IRMS with 16-bit ADC boards achieved high precision for less than 1 nmol of C on column, a common benchmark for GC–CIRMS applications, despite using 16-bit ADC boards. Lower inlet flow rates, enabling reduced pumping requirements, may be an important advantage in some applications.

References

- [1] W. Meier-Augenstein, *J. Chromatogr. A* 842 (1999) 351.
- [2] J.T. Brenna, T.N. Corso, H.J. Tobias, R.J. Caimi, *Mass Spectrom. Rev.* 16 (1997) 382.
- [3] M.P. Ricci, D.A. Merritt, K.H. Freeman, J.M. Hayes, *Org. Geochem.* 21 (1994) 561.
- [4] K.J. Goodman, J.T. Brenna, *J. Chromatogr. A* 689 (1995) 63.
- [5] K.J. Goodman, J.T. Brenna, *Anal. Chem.* 66 (1994) 1294.
- [6] K.J. Goodman, J.T. Brenna, *Anal. Chem.* 64 (1992) 1088.
- [7] G.L. Sacks, J.T. Brenna, J.T. Sepp, in: *Proceedings of the 49th ASMS Conference on Mass Spectrometry*, Chicago, IL, 2001.
- [8] National Instruments, Austin, TX, 2000.
- [9] N. Dyson, *Chromatographic Integration Methods*, Royal Society of Chemistry, Cambridge, UK, 1990.
- [10] J. Santrock, S.A. Studley, J.M. Hayes, *Anal. Chem.* 57 (1985) 1444.
- [11] D.A. Merritt, J.M. Hayes, *Anal. Chem.* 66 (1994) 2336.
- [12] S. Haykin, *An Introduction to Analog and Digital Communications*, Wiley, New York, 1989.

14 High-Precision Measurement of $^{13}\text{C}/^{12}\text{C}$ Ratios by On-Line Combustion of GC Eluates and Isotope Ratio Mass Spectrometry

M. RAUTENSCHLEIN, K. HABFAST AND W. BRAND

Finnigan MAT GmbH, Barkhausenstrasse 2, D-2800 Bremen, FRG

Introduction

The stable isotopes of carbon (^{12}C , ^{13}C) are widely used in natural sciences: in geology, by measuring isotope ratios; in organic compounds, or in human studies, by using artificially enriched compounds as tracers.

First, the natural variations of the $^{13}\text{C}/^{12}\text{C}$ ratio are studied by means of isotope ratio mass spectrometry (IRMS). Various processes fractionate the carbon isotopes in nature. The observed isotope ratios provide information on these processes, for example on palaeotemperatures or on specific chemical or biological reactions; or they can be used, for instance, to trace the origin and/or genesis of a specific compound.

Secondly, the isotopic content of ^{13}C in samples artificially enriched with ^{13}C -labelled components is measured, usually with combined gas chromatography/mass spectrometry (GC/MS). This replaces ^{14}C labelling and thus avoids any radiation hazards. This method is used preferentially in biology, biochemistry or clinical and environmental research, where medical ethics discourage the use of radioactive tracers for investigative purposes.

In both application areas very small amounts of ^{13}C must be detected. On the one hand, significant natural variations in the approximately 1.1% ^{13}C abundance (in relation to ^{12}C) are as small as 0.0002 atom%. Hence, small natural variations in the amount of ^{13}C must be detected in the presence of 100 times more ^{12}C . This requires very high precision in measuring the $^{13}\text{C}/^{12}\text{C}$ ratio, as is normal with IRMS.

On the other hand, in labelling studies, normally only very small amounts of sample are available and the compounds are separated on line in the GC/MS. Hence, the capability for detecting small amounts of ^{13}C is mandatory. From an analytical point of view, this is totally equivalent to the requirement for high

Stable Isotopes in Paediatric Nutritional and Metabolic Research
© Intercept Ltd, PO Box 716, Andover, Hampshire, SP10 1YG, UK

precision in the $^{13}\text{C}/^{12}\text{C}$ natural ratio determination in IRMS as only low amounts of ^{13}C -labelled material are present.

The type of compound (organic, inorganic, solid, liquid, gaseous) that must be handled by these mass spectrometric methods is very diverse. No mass spectrometer, IRMS or GC/MS is able to accept such a broad variety of sample types directly for an isotope ratio determination. In $^{13}\text{C}/^{12}\text{C}$ isotope ratio mass spectrometry each isolated compound of interest is combusted to CO_2 then measured. This greatly simplifies the mass spectrometer and offers two definite advantages towards the achievement of high precision and accuracy: the isotope ratio can always be measured by one and the same sample triple collector, adjusted for the simultaneous recording of masses 44 ($^{12}\text{C}^{16}\text{O}_2$), 45 ($^{13}\text{C}^{16}\text{O}_2$) and 46 ($^{12}\text{C}^{18}\text{O}^{16}\text{O}$) and one can always use the same inexpensive working standard gas (CO_2) to calibrate the mass spectrometer easily at short time-intervals.

This elegant methodological simplicity in IRMS is counterbalanced by the apparent need of a variety of sample preparation and isolation methods, which are more or less specially adapted to certain compound types. Such methods range from rather special ones, e.g. the reaction of carbonates with phosphoric acid to yield CO_2 , to more universally applicable processes, such as the catalytic combustion of all kinds of organic compounds. If the isotope ratio of one or of all compounds in a mixture is requested, a separation process (e.g. a gas chromatographic separation) is required before the conversion to CO_2 .

For all of these methods, there is one common requirement: they must separate and then convert the sample into CO_2 so that the isotope ratio of each sample is preserved in the CO_2 measured by IRMS. Apparently, this requirement is the critical step of the whole method.

Classical isotope ratio mass spectrometers use a dual gas-inlet system, where one sample container is used to store the sample CO_2 and the other one to store the working standard gas. Sample and standard, both pure CO_2 , are repeatedly introduced intermittently at 4–8 seconds' time-intervals into the mass spectrometer and are directly compared to each other.

By this (batch-) type of sample to standard comparison method, the highest possible precision and accuracy is achieved. However, the method is totally incompatible to modern types of continuous-flow separation, such as gas chromatography or combustion devices, which normally use a carrier gas like helium and which make each sample available for measurement as a 'peak' for a few seconds only.

This incompatibility led to the (recent) development of the non-classical continuous-flow introduction method, which will be described in more detail below. We call this method in its combination with a gas chromatograph, 'GC combustion isotope ratio mass spectrometry (GC/C/IRMS)'.

Experimental set-up of GC/C/IRMS

Figure 14.1 shows the schematic layout of our complete system: a capillary gas chromatograph is coupled on-line to a triple collector (masses 44/45/46) isotope ratio mass spectrometer via a combustion interface. A second, switchable gas inlet

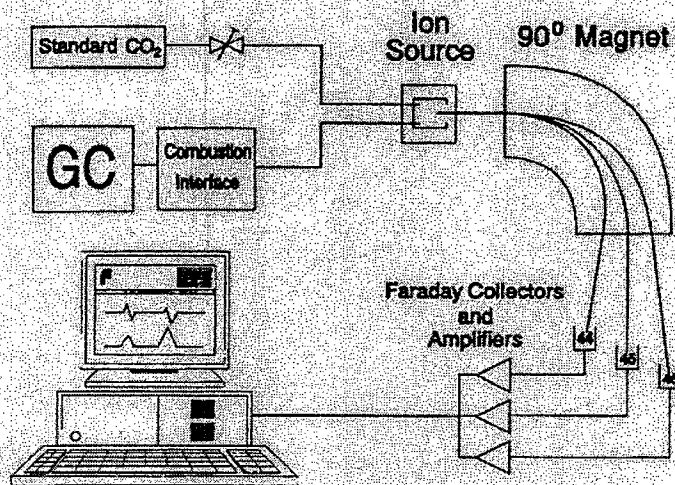


Figure 14.1 Schematic layout of the GC/C/IRMS (gas chromatograph/combustion/isotope ratio mass spectrometer) system.

line to the mass spectrometer is used to introduce a standard gas (CO_2). The mass spectrometric data are acquired and evaluated by a PC-based data system using a multitasking operating system with a graphic, menu-operated user interface.

In Figure 14.2, the combustion interface is shown in more detail. A capillary gas chromatographic column is connected to a high-efficiency microcombustion furnace via a post-column splitter. The second outlet of the splitter can be

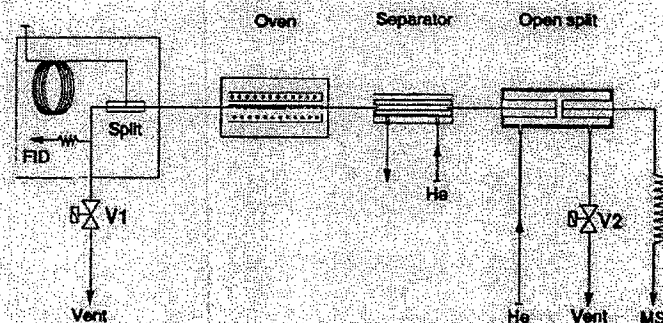


Figure 14.2 Combustion interface of the GC/C/IRMS system.

connected to any type of GC detector (flame ionization detector (FID), thermal conductivity detector (TCD) or ion trap detector/mass spectrometer (ITD/MS)) and is connected in parallel to a high-conductivity vent, which can be closed by valve V1.

The combustion furnace consists of a quartz capillary at 820°C, filled with platinum and copper oxide, serving as a combustion catalyst and as a source of oxygen. Behind the furnace, a capillary-shaped phase separator, made of selectively permeable membrane materials, is connected. This simple dead-space-free device removes all water from the mixture of combustion products. A conventional open split couples the combustion system to the mass spectrometer via a capillary, which limits the flow to 0.6 ml min⁻¹, practically independent of the flow conditions in the rest of the system. The open split is fed with a constant flow of make-up gas (He) which can leave the split device via valve V2.

The chromatographic resolution of the complete, virtually dead-space-free

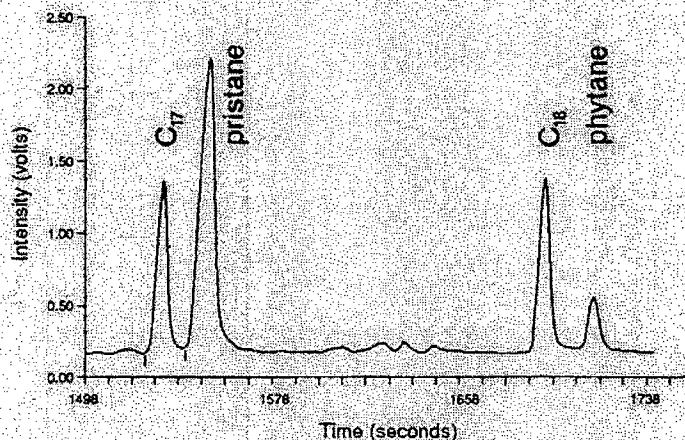


Figure 14.3 Detail of a combusted crude oil chromatogram: recording of the $^{13}\text{C}^{18}\text{O}_2^+$ ion current, m/z 44.

combustion system can be judged from Figure 14.3, which shows part of the mass 44 recording trace of a mixture of hydrocarbons.

Depending on the status of the two valves V1 and V2 (Figure 14.2), the combustion system operates in two different modes. First, during normal operation, when the GC eluates are to be combusted, V1 is closed and V2 is open. Thus, because of the closure of the high conductivity vent, the post-column split feeds the majority of the gas flow through the combustion system and only a small portion is flowing to the GC FID detector.

Secondly, in order to prevent the solvent, or any other undesirable eluate (which would possibly destroy the furnace's catalytic activity), from entering the combus-

tion system, V1 is opened and V2 is closed. In this way, the flow through the system is reversed by the open split's make-up gas, and all GC eluates are now completely directed to vent via the post-column split. The flow to the GC detector is still maintained, as well as the pure helium flow to the mass spectrometer.

For illustrative simplicity, another feature of the combustion system is not shown in *Figure 14.2*: oxygen can be introduced into the furnace to regenerate the copper oxide that is slowly used by the combustion process.

In order to cope with the relatively high helium flow, the mass spectrometer itself is differentially pumped. For the same reason the gas conductance of the ion source is raised to approximately 1.6 l s^{-1} (air), which results in approximately half the ion-source sensitivity as compared to the normal operation of an isotope ratio mass spectrometer (without helium flow): for 1 nmol of CO_2 introduced into the ion source, one detects about 8×10^{10} ions at the mass 44 collector.

For the measurement of an isotope ratio of 1 : 100, the statistical limit allows a best relative precision of approximately 5×10^{-3} for one nmol of CO_2 . In the Faraday cup recording system, the rise time of the DC amplifiers for the three ion collectors is carefully matched to an appropriate value [200 milliseconds (ms)], although they use working resistors which differ by more than a factor of 100 (because of the different abundances of the three ion currents). The ion current signals are integrated for selectable time-intervals (range 60–500 ms). Hence, at least 20 triple-collector readings can be taken for a 5 s GC peak.

Typical ion current (m/z 44) and isotopic ratio (m/z 45 : m/z 44) chromatograms are shown in *Figure 14.4*. The lower graph shows the intensity recording of the mass 44 ($^{12}\text{CO}_2$) trace, and is very similar to a direct FID trace of the gas chromatograph or a total ion current recording chromatogram of a conventional GC/MS system. The upper graph shows the plot of the ratios of the traces of masses 45 and 44 as a function of time. The scale is calibrated in δ -values, i.e. the relative difference of the measured ratio (in Pee Dee belemnite limestone) given in parts per thousand or per mil (‰). In addition, a total of three standard CO_2 gas injections are shown before and after peak number 4, benzaldehyde (*Figure 14.4*).

The isotope ratio traces of the gas chromatographic peaks exhibit a typical S-shape, whereas the standard gas injections, which are introduced directly into the ion source, show a nearly rectangular ratio response (*Figure 14.4*, upper trace). The reason for the S-shaped isotope ratio (*Figure 14.5*, lower trace) of the GC peak is that the 'heavier' isotopic species of a compound elutes more rapidly from the high-resolution capillary column than the light species (*Figure 14.5*, upper trace). This behaviour is the result of a vapour pressure isotope effect, and is an often observed phenomenon in GC/MS selected ion monitoring. The actual ratio is computed from the ratio of the area under the two isotopic peaks. Hence, care must be taken to integrate in currents across the full width of the chromatographic peaks.

During the run of a gas chromatogram, a real-time plot of the GC trace and of the $^{13}\text{C}/^{12}\text{C}$ ratio is shown on the screen of the computer. Data evaluation is performed fully automatically after the completion of the gas chromatogram. The user can manually change the integration program via a dialogue, if necessary, to advise the system of the proper choice of baseline ratio values.

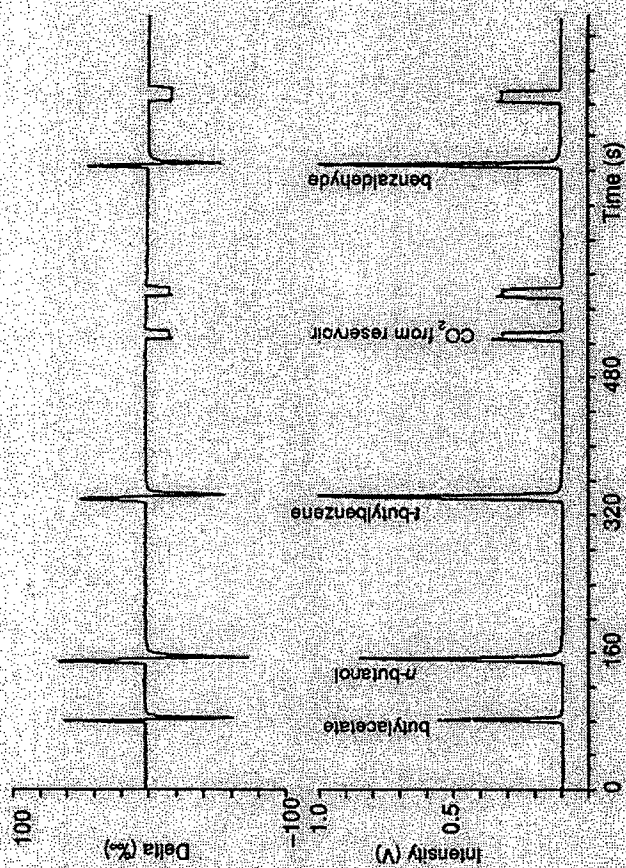


Figure 14.4 Chromatogram ($^{13}\text{C}/^{12}\text{C}$ ion current, m/z 44) and isotopic ratio (in ‰ notation) of a standard mixture, together with three CO_2 reference gas injections.

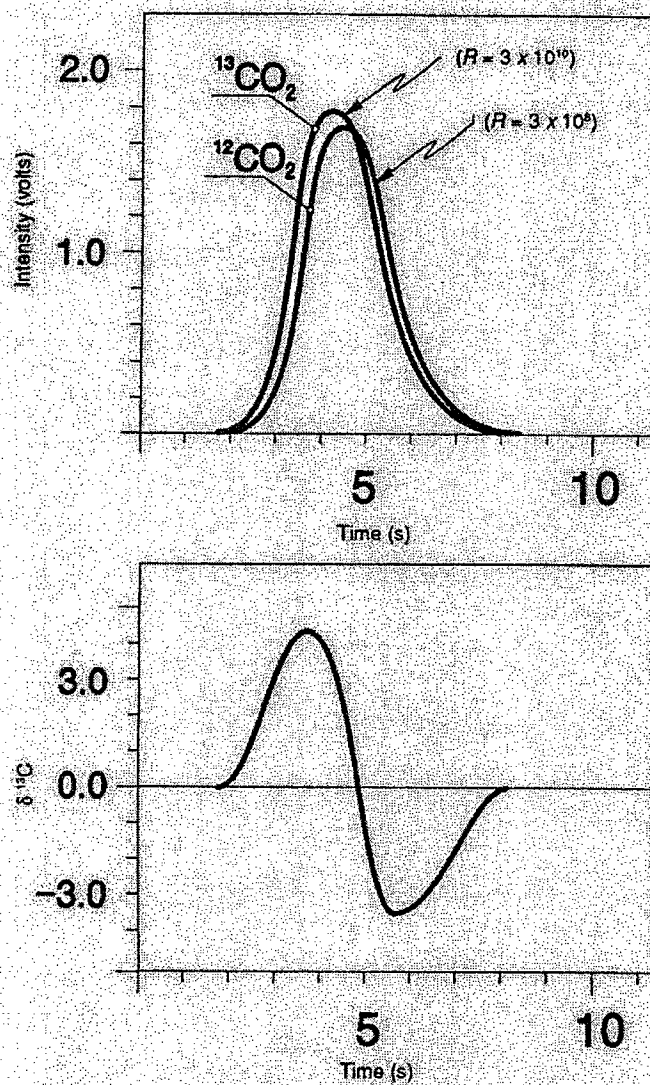


Figure 14.5 The origin of the S-shaped ratio output: the heavier isotopic species elutes more rapidly from the capillary column than the lighter (more abundant) ^{12}C species.

Nomenclature and methodology

The 'isotope ratio' (r) of a sample is the ratio of the number (n_i) of two isotopes ($i = 1, 2$):

$$r = \frac{n_2}{n_1}$$

Therefore, the atom% value (a) of the (n_2) isotope is:

$$a = \frac{100 r}{(r + 1)} = \frac{(100 n_2)}{n_1 + n_2}$$

The 'enrichment' of an isotope in a sample as compared to a standard value (a_s) is, therefore, given by atom% excess (APE):

$$\text{APE} = a - a_s$$

An isotope ratio mass spectrometer measures the isotope ratio. The measured raw isotope ratio is, however, influenced by several mass discriminating effects which fluctuate with time and from instrument to instrument. In order to achieve the best precision and the best accuracy, each ratio measurement *must* be calibrated by the measurement of a standard with known isotopic composition. Otherwise, measurements made at different times on the same instrument and other instruments would be incomparable.

This is the basic reason why isotope ratios are always measured and reported relative to a standard value. However, classically with IRMS (and in contrast to the usual way to report the amount of isotopically labelled species in a sample, as with GC/MS) not the 'enrichment' versus a standard, i.e. the value APE (where r_s is the isotope ratio in the standard)

$$\text{APE} = \frac{(r_s - r) 100}{(r + 1)(r_s + 1)}$$

is reported, but the so-called δ -value:

$$\delta = \frac{r}{r_s - 1} \times 1000$$

This is the relative difference of a ratio r from a standard ratio r_s , given in parts per thousand, or per mil (‰).

The relationship between the enrichment and the δ -value is basically non-linear. For all details, how to 'convert' δ -values into atom% or atom% excess values and vice versa, see Figure 14.6.

If the measured isotope ratios are related to a standard near the natural level of ^{13}C (approximately 1.1‰), a linear approximation can be used for δ -values up to approximately $\delta = 1000$ ‰ (i.e. for $r = 2r_s$ or ≈ 1 atom% excess):

$$\text{APE} = \frac{a_s \times \delta}{1000}$$

$$\text{ape}_u = a_s \frac{\delta_u \times (100 - a_s)}{a_s \times \delta_u + 10^5}$$

$$a_u = a_s \frac{(1 + \frac{\delta_u}{1000}) \times 10^5}{a_s \times \delta_u + 10^5}$$

$$\delta_u = \frac{\text{ape}_u \times 10^5}{100 - (1 + \text{ape}_u) \times a_s}$$

$$\delta_u = \frac{(a_u - a_s) \times 10^5}{(100 - a_u) \times a_s}$$

a_u, a_s, ape_u in %

δ in ‰

Figure 14.6 The conversion of atom% values into δ -values and vice versa. a_u , atom% of sample; a_s , atom% of standard; ape_u , atom% excess in sample.

This relationship shows the 'high resolution' of the δ -scale as compared to the atom% scale: a δ -value of 1‰ corresponds to 0.001 atom% excess at natural level (1%).

Table 14.1 Reproducibility of the GC/C/IRMS system; the samples were measured over 6 weeks

| Measurement | <i>n</i> -Butylacetate $\delta^{13}\text{C}(\text{‰})$ | <i>n</i> -Butanol $^{13}\text{C}/^{12}\text{C}$ ratio (%) | <i>t</i> -Butylbenzene $\delta^{13}\text{C}(\text{‰})$ | 5-Nonanone $\delta^{13}\text{C}(\text{‰})$ | Benzaldehyde $\delta^{13}\text{C}(\text{‰})$ |
|-------------|---|---|---|---|---|
| 1 | -5.50 | 1.15626 | -1.24 | | |
| 2 | -5.49 | 1.15636 | -0.84 | | |
| 3 | -6.06 | 1.15625 | -1.01 | | |
| 4 | -5.42 | 1.15486 | -1.05 | | |
| 5 | -5.05 | 1.15346 | -0.80 | -4.25 | |
| 6 | -5.32 | 1.15308 | -0.58 | | |
| 7 | -5.33 | 1.15443 | -0.35 | | |
| 8 | -5.36 | 1.15547 | -0.94 | | |
| 9 | -5.51 | 1.15660 | -0.84 | | |
| 10 | -5.36 | 1.15683 | -0.97 | -4.76 | |
| 11 | -5.24 | 1.16439 | -0.37 | | |
| 12 | -5.66 | 1.16277 | -1.09 | | |
| 13 | -5.14 | 1.15995 | -1.57 | | |
| 14 | -5.67 | 1.15955 | -0.97 | | |
| 15 | -5.18 | 1.15900 | -0.71 | | |
| 16 | | 1.15779 | -1.07 | | |
| 17 | -5.18 | 1.15664 | | | -2.82 |
| 18 | -5.75 | 1.15485 | -1.11 | | -2.89 |
| 19 | -5.14 | 1.15492 | -0.80 | | -2.46 |
| 20 | -5.58 | 1.15494 | -1.31 | | -2.75 |
| 21 | -5.46 | 1.15720 | -1.11 | | -2.63 |
| 22 | -5.21 | 1.15993 | -0.80 | | -2.39 |
| 23 | -5.51 | 1.15987 | -1.32 | -4.59 | -2.88 |
| Mean | -5.41 | 1.15736 | -0.95 | -4.53 | -2.69 |
| SD | 0.23 | 0.00261 | 0.29 | 0.21 | 0.19 |

n-Butanol was used as the internal standard.

For a complete GC run, basically two different ways of standardization are available. First, a dose of CO_2 of known isotopic composition is introduced directly into the ion source (or somewhere into the helium flow of the combustion interface) and the isotopic ratio of all GC peaks is related to this gas. Several standard injections can also be distributed across the whole run, if necessary.

Secondly, one or several compounds with known isotopic composition are used as internal standards in the sample mixture. Besides the proper use of a standard, the selection (or measurement) of the correct baseline values is important. It must be noted that the isotope ratio of the baseline may be different at different times during the GC run, due to a changing column bleed.

Table 14.1 summarizes the repeated measurements of the mixture of standards that was shown in Figure 14.4. *n*-Butanol was used as internal standard for measurement of the isotope ratios. The external standard deviation of these measurements is better than 0.3‰. This means that two samples whose isotopic composition differs by only 0.0003 atom% can be differentiated by this method.

In order to demonstrate the excellent linearity of the method over a wide range of (small) isotopic concentrations, we have measured several precise mixtures of $[1-^{13}\text{C}]$ palmitate diluted with unlabelled palmitate as methyl ester derivatives. Our results are shown in Figure 14.7. The measured δ -values have been converted into atom % excess values, using the isotopic content of the unlabelled palmitate methyl ester as the reference. It should be noted that a range of approximately 7‰ in the δ -value covers almost two orders of magnitude in isotopic concentration of the sample.

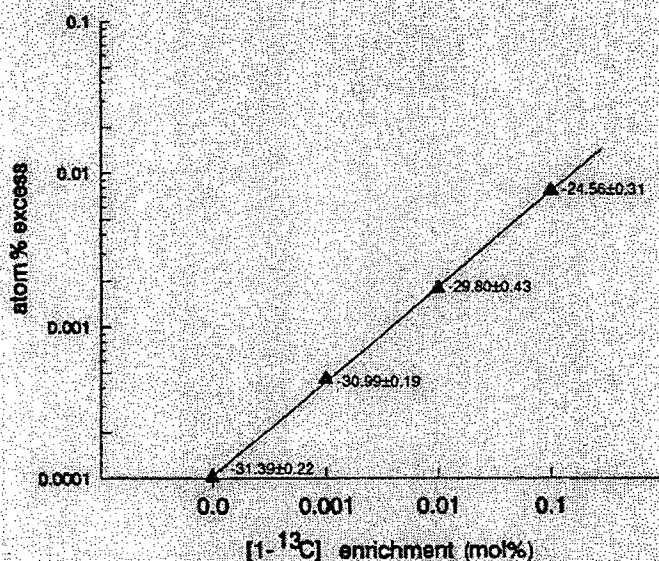


Figure 14.7 Calibration graph of a $[1-^{13}\text{C}]$ palmitate methyl ester dilution series. The measured δ -values (\pm SD), shown next to the data points, have been converted into atom % excess values. (Samples courtesy of T.E. Chapman, University Hospital Groningen, The Netherlands.)

In order to check not only the precision, but also the accuracy of our results (i.e. of our standardization procedure), the individual compounds of the test mixture (Figure 14.4) were analysed individually with the classical high-precision total combustion method (Dumas combustion). The mean isotope ratios (and their standard deviations) are shown in Table 14.2, together with the means from the GC combustion run of the mixture. The difference of the isotopic ratios obtained by both methods is smaller than 0.2‰ (δ -notation) in most cases and is, therefore, safely within the statistical uncertainties.

Table 14.2 Comparison of GC/C/IRMS and classical off-line combustion and IRMS isotope analysis of a standard mixture

| Compound | GC/C/IRMS $\delta^{13}\text{C}_{\text{PDB}}$ (‰) mean (\pm SD) | Dual inlet $\delta^{13}\text{C}_{\text{PDB}}$ (‰) mean (\pm SD) | Difference in $\delta^{13}\text{C}$ (‰) |
|------------------------|---|--|--|
| <i>n</i> -Butylacetate | -28.97 (0.23) | -28.78 (0.01) | 0.19 |
| <i>n</i> -Butanol | -23.56 (0.06) | -23.56 (0.06) | (0.00) |
| <i>t</i> -Butylbenzene | -24.51 (0.29) | -24.50 (0.02) | 0.01 |
| 5-Nonanone | -28.06 (0.21) | -28.48 (0.02) | 0.42 |
| Benzaldehyde | -26.23 (0.19) | -26.15 (0.05) | 0.08 |

n-Butanol was used as the internal standard in the GC/C/IRMS measurements, its $\delta^{13}\text{C}$ value was measured using off-line combustion and classical IRMS.

The difference in $\delta^{13}\text{C}$ (‰) values between the two techniques was GC/C/IRMS values minus the dual inlet values.

PDB, Pee Dee belemnite limestone.

Application examples

The carbon and hydrogen isotopic ratios of the light hydrocarbons in natural gas are useful indicators for gas and oil deposits. *Figure 14.8* shows a typical recording of the measurement of a natural gas sample, together with two reference CO_2 gas injections at the end of the gas chromatogram. The δ -values range from -32‰ to -25‰, with standard deviations in measurements ranging from 0.2‰ to 0.4‰ ($n = 8$) (*Figure 14.9*).

The *n*-alkanes in crude oil also show differences in isotopic ratios. An example for *n*-alkane up to C_{30} is shown in *Figure 14.10*.

Food flavours are normally mixtures of compounds. For economic reasons, very often such flavours are synthesized artificially instead of being extracted from plants. Synthesized flavour extracts show a different isotopic pattern from their natural counterparts, and this can be used to differentiate between them. One example (strawberry aroma) is given in *Figure 14.11*.

An interesting feature of the GC combustion isotope ratio method is shown in *Figure 14.12*. It shows a gas chromatogram of an extract from a fermentation experiment. At two locations in the chromatogram one can observe a big (non-S-shaped) isotope signal (upper trace) whereas virtually no corresponding peak can be found at m/z 44 (lower trace). This points to a highly ^{13}C -enriched compound (virtually no ^{12}C content) at a very low concentration.

In *Figure 14.13*, we therefore show the enlarged traces of masses 44 and 45: a $^{13}\text{C}^{16}\text{O}_2^+$ trace can be detected (10 mV), a $^{12}\text{C}^{16}\text{O}_2^+$ trace at natural level should be present with about the same intensity, but is, actually, much smaller. This is the

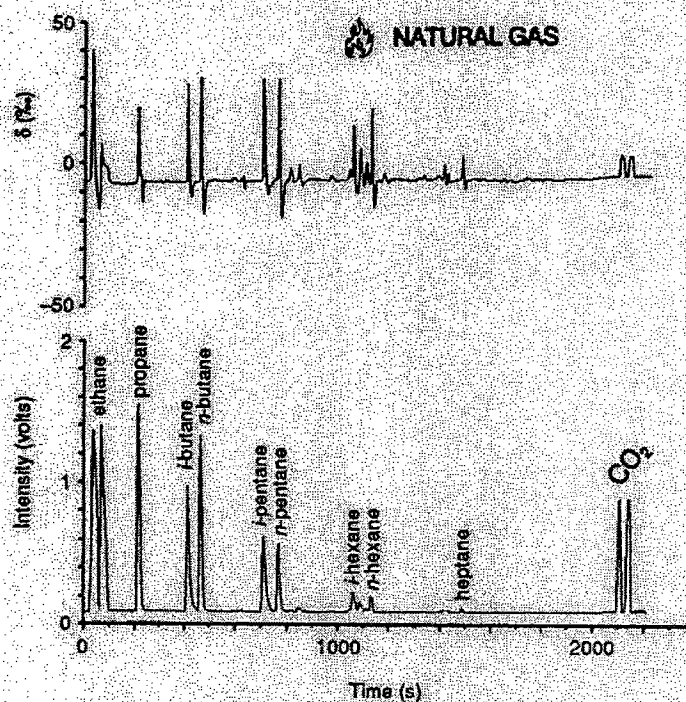


Figure 14.8 Raw data output from a natural gas sample: $^{13}\text{C}^{16}\text{O}_2^+$ ion current, m/z 44, and isotopic ratio (δ -notation).

explanation why the ratio signal does not show the typical S-shape. The isotopic content (corrected for baseline) is 4 atom% excess. The peak height at mass 45 corresponds to 2 pg of ^{13}C label, the signal-to-noise ratio of the ratio peak being better than 100 : 1 (Figure 14.13, upper trace). The 'detection limit' with a signal-to-noise ratio of 10 : 1 would, therefore, be estimated to be 200 fg.

With these few examples we have demonstrated the great potential of this new analytical method. We believe it will find many applications in the future.

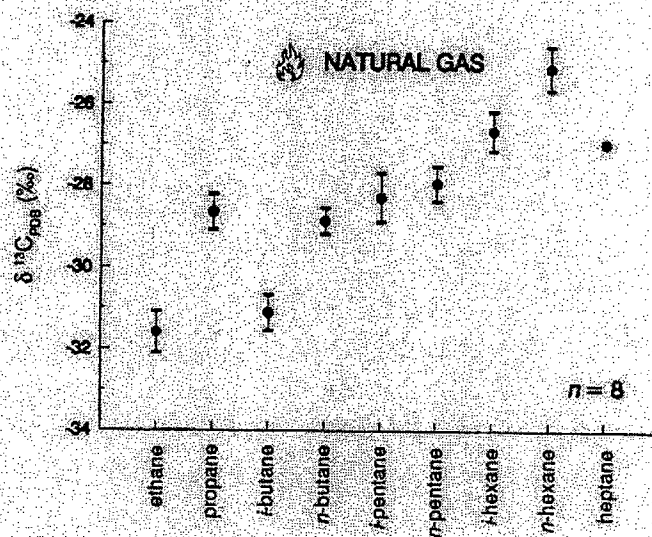


Figure 14.9 The isotope ratios of a natural gas sample, relative to Pee Dee belemnite limestone (PDB), the international standard.

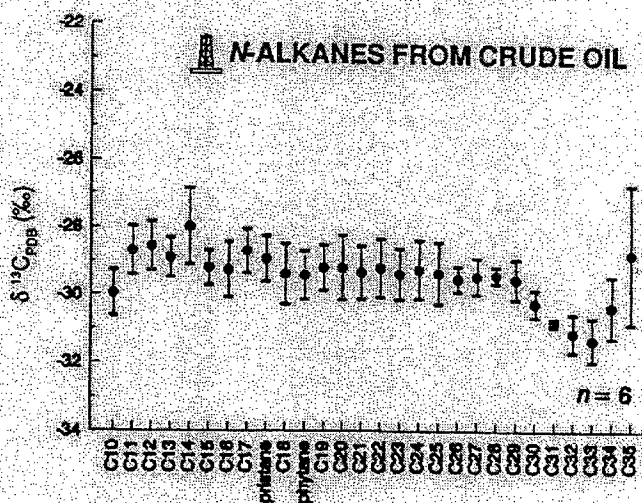


Figure 14.10 Isotopic pattern of the *n*-alkanes of a crude oil sample in the range C10–C35.

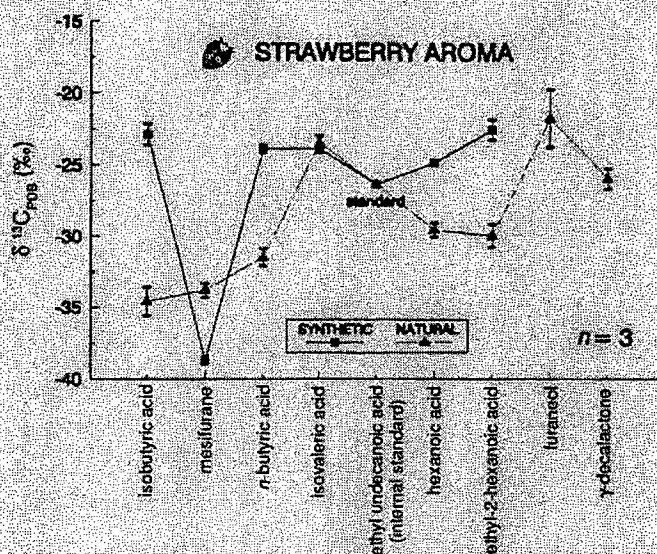


Figure 14.11 Isotopic pattern of compounds in a natural and synthetic strawberry aroma (courtesy of J. Koziet, Centre de Recherche Pernod Ricard, Créteil, France).

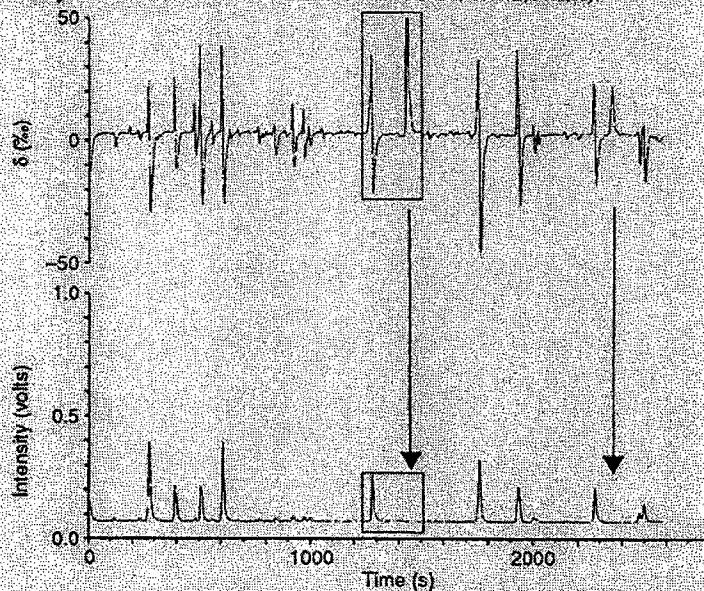


Figure 14.12 Raw data output from a mixture of metabolites. Boxes show area of chromatogram displayed in detail in Figure 14.13.

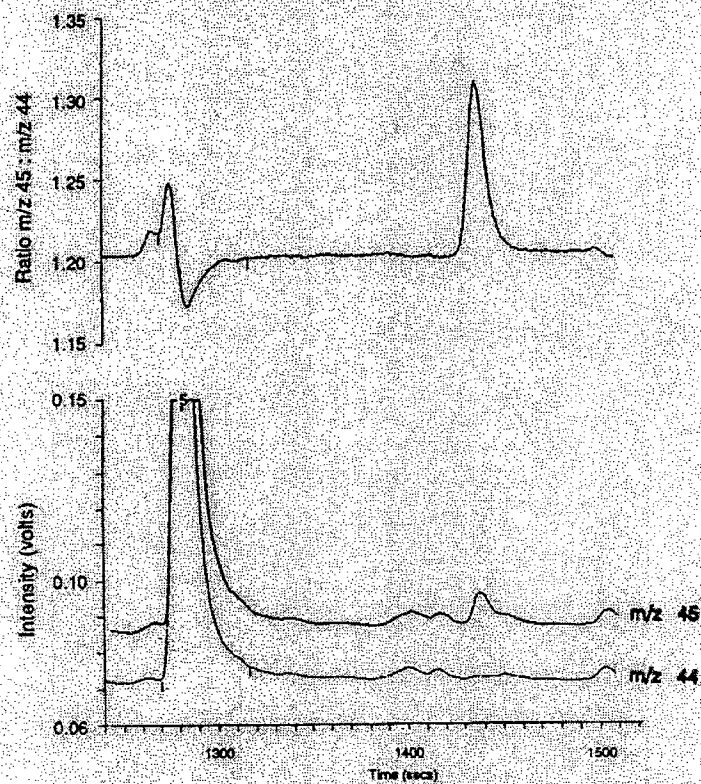


Figure 14.13 Enlarged display of a portion of the small peak observed in Figure 14.12 (see boxes) with $^{13}\text{C}^{16}\text{O}_2$ ($m/z = 45$) and $^{13}\text{C}^{18}\text{O}_2$ ($m/z = 44$) traces. (The amplification of the signal from the m/z 44 ion is 100 times less than that of the m/z 45 ion.)

Some instrumental effects in the determination of stable carbon isotope ratios by gas chromatography–isotope ratio mass spectrometry

P.A. Eakin, A.E. Fallick and J. Gerc

Isotope Geology Unit, Scottish Universities Research and Reactor Centre, East Kilbride, Glasgow, G75 0QU, Scotland, UK

(Received March 11, 1991; revised and accepted July 8, 1991)

ABSTRACT

Eakin, P.A., Fallick, A.E. and Gerc, J., 1992. Some instrumental effects in the determination of stable carbon isotope ratios by gas chromatography–isotope ratio mass spectrometry. In: R.S. Harmon and R.W. Hinton (Guest-Editors), *Frontiers in Isotope Geosciences, Chem. Geol. (Isot. Geosci. Sect.)*, 101: 71–79.

Some sources of instrumental errors in the determination of $^{13}\text{C}/^{12}\text{C}$ in organic compounds by gas chromatography–isotope ratio mass spectrometry (GC–IRMS) have been investigated. For mass 44 ion beam intensities in the range $1 \cdot 10^{-10}$ to $1 \cdot 10^{-8}$ A, mass-spectrometric pressure effects do not significantly affect data accuracy, thus obviating the necessity of matching sample and reference ion beam intensities for each compound in a complex sample. Data quality is influenced by: (a) the quality of the furnace-tube packing; and (b) the performance of the cryogenic trap. On analysis of large (70 ng) samples, precisions (1σ) of $\leq \pm 0.23\%$ were obtained using 0.35–0.5-mm-grade CuO. However, there was a marked deterioration in precision ($1\sigma \leq \pm 0.62$) when coarse CuO (0.35–1 mm grade) was tested. Use of fine CuO also permits analysis of smaller samples. Inefficient trapping, and release of water during overnight defrosting leads to erroneously low $\delta^{13}\text{C}$ -values, accuracies of -1.1 to -0.25% resulting from analysis of 70-ng samples. Accuracy error increases to $\leq 1.86\%$ on analysis of 10-ng samples. Efficient water trapping combined with the improvements in furnace performance results in precisions generally much better than $\pm 0.4\%$ and accuracies better than $\pm 0.65\%$ for high-molecular-weight compounds (268–338 amu), and very accurate and precise results (better than $\pm 0.2\%$) for lower-molecular-weight compounds (142–173 amu).

1. Introduction

The VG Isochrom II[®] gas chromatography–isotope ratio mass spectrometer (GC–IRMS) system allows rapid determination of $\delta^{13}\text{C}$ -values of individual organic compounds in complex mixtures. The method utilises a gas chromatograph to separate the sample into its constituent compounds (Fig. 1a). These compounds elute from the column-end at different times according to their molecular weight and structure.

Correspondence to: P.A. Eakin, Isotope Geology Unit, Scottish Universities Research and Reactor Centre, East Kilbride, G75 0QU, Scotland, UK.

On elution, the compounds are carried in a continuous helium stream through an oxidising furnace containing granules of Cu(II)O (Fig. 1b). The rate of combustion and passage through the furnace is similar for all compounds, thus the separation achieved during chromatography is maintained. H_2O is removed from the oxidation products by means of a cryogenic trap (Fig. 1c). The mixture then passes to a single-inlet, triple-collector isotope ratio mass spectrometer (Fig. 1d). The stable carbon isotope composition of each compound is computed by comparison of the sample 45/44 ratio with that of reference CO_2 (Fig. 2) and normal corrections are applied for the

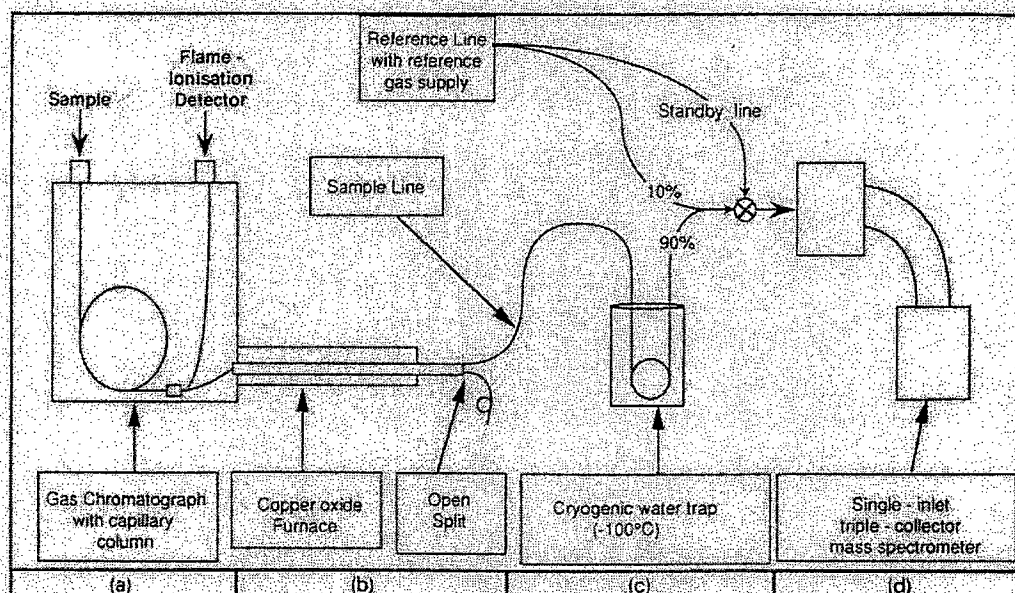


Fig. 1. Schematic representation of the VG Isochrom® GC-IRMS system.

- The gas chromatograph is responsible for separation of the mixture into its constituent compounds.
- The furnace consists of a 0.63-mm-ID quartz tube filled with CuO. An outer element heats the tube to 850°C. The organic compounds are swept through the tube in a He stream.
- Passage from the furnace to the mass spectrometer is controlled by the pressure drop to the spectrometer vacuum system. The temperature-controlled cryogenic trap is set to -100°C to remove water from the combustion products.
- The mass spectrometer is a single-inlet, triple-collector instrument. δ -values are determined by comparison of the m/e 45/44 ratios of the sample and reference gases. Reference pulses are distributed around the sample peaks (e.g., see Fig. 2).

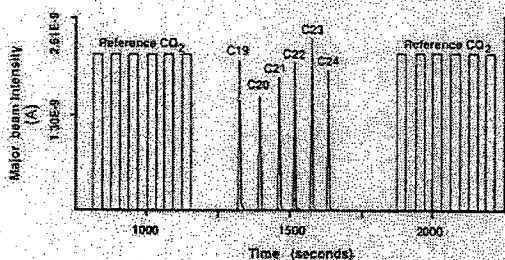


Fig. 2. Idealised GC-IRMS trace for standard mixture B. Flat-topped peaks indicate reference gas pulses, sharp peaks represent C_{19} – C_{24} *n*-alkanes.

mass spectrometric results (Craig, 1957).

The GC-IRMS technique was first developed by Sano et al. (1976) for the detection of

^{13}C -enriched metabolites from human patients. An improved technique was described by Matthews and Hayes (1978), who achieved precisions better than $\pm 0.5\%$ for both $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ in CO_2 and N_2 , and were able to detect enrichments of the heavy isotope as low as 0.004% excess. The advent of commercially available GC-IRMS systems capable of determinations of $^{13}\text{C}/^{12}\text{C}$ at natural abundance levels (there is also a conceptually similar Finnigan® instrument) realises an opportunity to develop an entirely new field of research into the distribution of carbon isotopes in organic matter. However, the general availability of such technology also necessitates a thorough assessment of errors induced by the combination of the GC, interface and mass spectrometry.

ter, and the utilisation of a continuous flow system.

Here we report the initial results of investigations into some potential instrumental effects influencing the specific GC-IRMS system in use at SURRC. The tests involved analysis of two standard mixtures: A, containing decane, undecane, dodecane and methyl-decanoate; and B, composed of C_{19} to C_{24} *n*-alkanes. The isotopic compositions of the standard compounds were determined by the conventional sealed-tube combustion technique, to precisions better than $\pm 0.1\text{‰}$ (1σ). Mixture A was injected using the split injection method where the bulk of the sample is lost through a split vent, and only a small proportion (3% during this investigation) enters the chromatography column. Mixture B was injected in splitless mode, where the split vent is closed and a large proportion of the sample enters the column. Experiments have shown that there is no isotopic discrimination associated with split injection (SURRC, unpublished data, 1990). $30\text{ m} \times 0.22\text{ mm ID OV1}^\circ$ (non-polar) and DB5 $^\circ$ (medium polarity) columns were used during the course of the investigation. Furnace tube characteristics were also changed as explained on p.76.

2. Sample/reference imbalance

One obvious potential source of error arises from differences in the maximum major beam intensity (^{44}I) between the reference and sample gases. In conventional isotope ratio mass spectrometry $^{44}\text{I}_{\text{sample}} = ^{44}\text{I}_{\text{reference}}$ thus obviating pressure effects, etc. In complex samples, composed of compounds with differing relative concentrations, sample/reference beam balancing is not possible for all components. To assess the effects of sample/reference mismatch, the $^{44}\text{I}_{\text{sample}}/^{44}\text{I}_{\text{reference}}$ ratio (S/R), was varied by analysis of increasing amounts of mixture A at fixed reference gas pressures. The results show that for $S/R < 0.5$ (injection of $< 1500\text{ ng}$ of sample at 40:1 split ratio), the

error in accuracy ($\Delta^{13}\text{C} = \delta_{\text{measured}} - \delta_{\text{known}}$) reaches almost -3‰ (Fig. 3). For $S/R > 0.5$, $\delta^{13}\text{C}$ remains consistent, yielding accuracies of $\leq 0.55\text{‰}$ and precisions (1σ) $\leq 0.63\text{‰}$. Fig. 3 implies that in the analysis of complex mixtures, accurate determinations on small peaks may be difficult to achieve. The possible reasons for the trends observed in Fig. 3 are discussed below.

2.1. Pressure effects

Pressure effects are manifest as an interdependence of the ion-beam intensities ratio (45/44) with ^{44}I (Craig, 1957). The effect has been attributed to a variety of factors especially contribution of ^{44}I to the minor collector as a result of peak broadening. For example, Craig (1957), in assuming a functional dependence proportional to $(^{44}\text{I})^2$, proposed a Coulombic effect within the major ion beam. Mook and Grootes (1973) claimed that peak broadening was due to ion scattering only. Fallick and Baxter (1977) have demonstrated a linear correlation between the major beam tail contribution to the minor beam collector and the abundance of target molecules available for collision within the spectrometer. The reliance of the Isochrom $^\circ$ on a He stream necessitates tolerance of a plethora of target molecules in the analyser, thus enhancing peak broadening. If pressure effects can be caused by peak broadening and if $^{44}\text{I}_{\text{sample}} \ll ^{44}\text{I}_{\text{reference}}$, variation in contributions of mass-44 ions to the minor beam could result in the computation of low sample δ -values.

However, Fig. 4 indicates that for ^{44}I from $1.5 \cdot 10^{-10}$ to $1 \cdot 10^{-8}\text{ A}$ there is no significant interdependence between the reference gas 45/44 ratio and ^{44}I (there is a slight increase in 45/44, where $^{44}\text{I} > 1 \cdot 10^{-8}$). Further, Fig. 3 indicated that there is no corresponding increase towards positive $\Delta^{13}\text{C}$ -values when $S/R > 0.5$ as might be expected if a significant pressure effect was influencing the results. Our results support the statement of Fallick and Baxter (1977) that overlap of the major beam into the

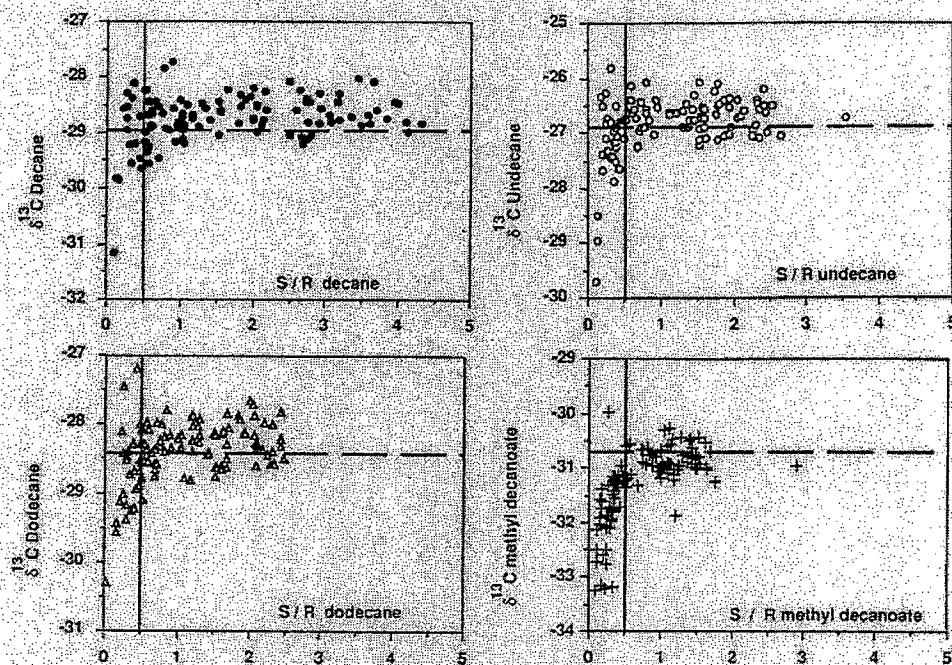


Fig. 3. $\delta^{13}\text{C}$ (‰ vs. PDB) of compounds in mixture A, plotted against S/R peak height ratio. Solid vertical line indicates $S/R=0.5$; dashed horizontal line indicates known isotopic composition (by conventional analysis). It is apparent from this figure that careful consideration must be given to accuracy, i.e. the relationship between δ_{isochrom} and δ_{known} for a given compound. A systematic offset in accuracy, whose origin is at present unknown, is noted for peaks 1–3.

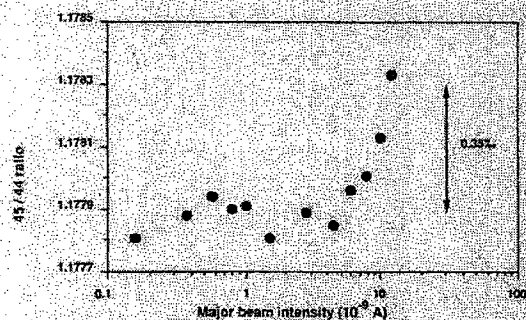


Fig. 4. Reference gas 45/44 ratio plotted against major beam intensity. The total variation could account for errors $\leq \pm 0.5\%$.

minor collector could not be responsible for pressure effects under the conditions of their experiment, as a purely additive component to the minor beam results.

We conclude that sample/reference imbalance is not responsible for the diminution in $\delta^{13}\text{C}$ evident in Fig. 3. Therefore, the results must be attributed to an instrumental effect associated with small sample analysis, or to the presence of contamination in the spectrometer source. The latter may be related to efficiency of the cryogenic trap, which is responsible for removal of impurities from the sample gas stream.

3. Sample gas purification

Removal of water from the sample He flow is achieved by controlled cooling of the combustion products to -100°C . The cold trap consists of a coil of $\frac{1}{32}$ -in (~ 0.8 mm) stainless-steel capillary wound round the top of a copper pipe. The other end of the pipe is sub-

merged in liquid nitrogen. The capillary is heated to -100°C by means of a heated coil wound outside the capillary. The temperature is controlled by a thermocouple positioned between the steel capillary and the copper pipe. Even distribution of the heat from the element is achieved through a conductive layer of copper tape wound between the steel capillary and the heated coil.

We have found that incorrect positioning of the heating element and thermocouple in the water trap assembly can lead to inefficient trapping of water. Furthermore, defrosting of the water trap overnight releases the trapped water. Although most will be lost to the vacuum pumps, some coats the capillaries to the mass spectrometer, and thereafter can enter the spectrometer source. The presence of water in the source is marked by an increase in the reference gas 45/44 ratio. However, during the course of a day's operation a systematic decrease in 45/44 ratio is observed as the water clears from the capillary (Fig. 5). When water trapping is grossly inefficient, the reference gas 45/44 ratio increases markedly. The exact mechanism by which this increase is achieved is unclear at the moment. However, the practical effect evident from our analysis is that low $\delta^{13}\text{C}$ of sample peaks result (Fig. 6). Furthermore, there is a positive linear correlation between the isotopic composition of consecutive

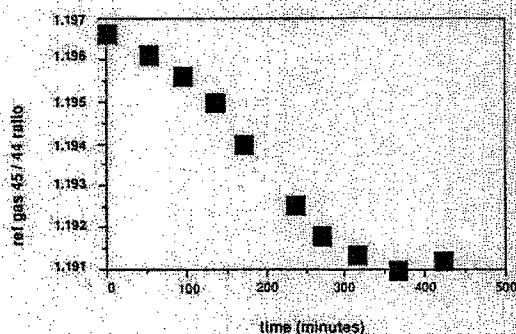


Fig. 5. Systematic variation in reference gas 45/44 ratio with time as a result of gradual clearing of water from the sample line capillary.

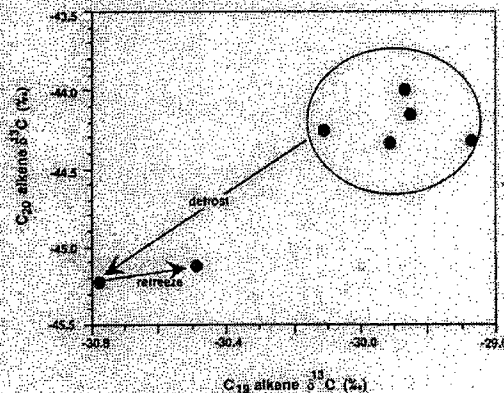


Fig. 6. Graphical representation of the effects of water-trap defrost on $\delta^{13}\text{C}$ -values of C_{19} and C_{20} *n*-alkanes in mixture B.

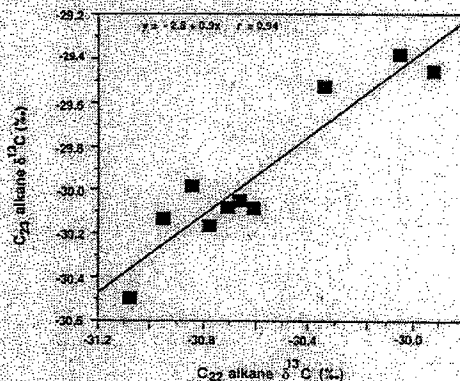


Fig. 7. C_{22} $\delta^{13}\text{C}$ vs. C_{23} $\delta^{13}\text{C}$ *n*-alkanes in mixture B for 10 determinations.

compounds eluting from the GC column (Fig. 7). This relationship holds for all peaks in both standard mixtures, and may result in the final peak having the greatest inaccuracies (always negative) of all peaks analysed (Table 1). To minimise such effects we recommend strict adherence to the water-trap structure now adopted by the manufacturers, and daily baking of the trap to prevent release of water to the capillaries during overnight defrosting.

Although anomalously negative $\delta^{13}\text{C}$ -values in sample peaks result, it is difficult to envisage how inefficiency in water trap perform-

TABLE I

Statistical analysis of repeated determinations of mixture B with varying furnace-tube and water-trap conditions

| Compound | C ₁₉ | C ₂₀ | C ₂₁ | C ₂₂ | C ₂₃ | C ₂₄ |
|---|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| <i>Conditions</i> —Sample=10 ng; CuO=0.35–1 mm grade; water trap inefficient: | | | | | | |
| Mean | –29.96 | –44.67 | –28.93 | –30.75 | –29.99 | –28.75 |
| <i>on</i> (<i>n</i> =13) | 0.59 | 0.89 | 0.70 | 0.44 | 0.48 | 0.60 |
| <i>d</i> | –0.72 | –0.22 | –1.48 | –1.38 | –1.19 | –2.21 |
| <i>Conditions</i> —Sample=70 ng; CuO=0.35–1 mm grade; water trap inefficient: | | | | | | |
| Mean | –30.49 | –45.29 | –28.76 | –30.59 | –29.93 | –28.30 |
| <i>on</i> (<i>n</i> =10) | 0.35 | 0.62 | 0.54 | 0.36 | 0.34 | 0.41 |
| <i>d</i> | –1.25 | –0.84 | –1.31 | –1.22 | –1.13 | –1.86 |
| <i>Conditions</i> —Sample=10 ng; CuO=0.35–0.5 mm grade; water trap inefficient: | | | | | | |
| Mean | –29.88 | –44.37 | –28.29 | –30.19 | –29.42 | –27.92 |
| <i>on</i> (<i>n</i> =12) | 0.18 | 0.38 | 0.40 | 0.32 | 0.27 | 0.41 |
| <i>d</i> | –0.64 | –0.08 | –0.84 | –0.82 | –0.62 | –1.38 |
| <i>Conditions</i> —Sample=70 ng; CuO=0.35–0.5 mm grade; water trap inefficient: | | | | | | |
| Mean | –29.93 | –44.18 | –28.38 | –30.04 | –29.38 | –27.64 |
| <i>on</i> (<i>n</i> =11) | 0.17 | 0.23 | 0.15 | 0.19 | 0.17 | 0.22 |
| <i>d</i> | –0.69 | –0.25 | –0.93 | –0.67 | –0.57 | –1.10 |
| <i>Conditions</i> —Sample=70 ng; CuO=0.35–0.5 mm grade; water trap efficient: | | | | | | |
| Mean | –29.32 | –43.82 | –27.51 | –29.58 | –28.74 | –26.96 |
| <i>on</i> (<i>n</i> =5) | 0.13 | 0.29 | 0.40 | 0.21 | 0.23 | 0.16 |
| <i>d</i> | –0.08 | 0.63 | –0.06 | –0.21 | 0.06 | –0.42 |

on=precision; *d*=accuracy error. Sample sizes refer to quantities of individual compounds.

ance could lead to the large errors evident in Fig. 3. Therefore, we assume that the errors generated where $S/R < 0.5$ are due to sample size constraints.

4. Furnace performance and sample size constraints

$S/R < 0.5$ (Fig. 3) corresponds to sample sizes decreasing from 1500 to 50 ng. To investigate data variability with sample size, multiple analyses of mixture B were performed. Splitless injection was used in an attempt to ensure consistent sample sizes were analysed. During the experiment the furnace tube packing was varied to gain information on conditions for optimum performance.

A furnace tube (600 mm × 0.65 mm ID) was

filled with coarse (0.35–1 mm grade) wire from copper oxide granules. The coarse filling was chosen to provide a low surface area for combustion, combined with high gas permeability and velocity through the furnace tube. Thirteen determinations on 10-ng aliquots of each *n*-alkane in mixture B (Table I) yielded accuracies generally better than $\pm 1.5\%$ (worst case $\pm 2.2\%$) and precisions generally better than $\pm 0.6\%$ ($\pm 0.89\%$ worst case). The experiment was then repeated with injection of 70-ng samples (corresponding to $S/R > 0.5$ in Fig. 3). The results (Table I) show improved precision ($\leq \pm 0.62\%$) and accuracy ($\leq \pm 1.25\%$ for peaks 1–5 and $\pm 1.86\%$ for peak 6).

Plotting the total variance ($\sum d^2 C_{C_{19}-C_{24}}$) against the weighted bulk isotopic composi-

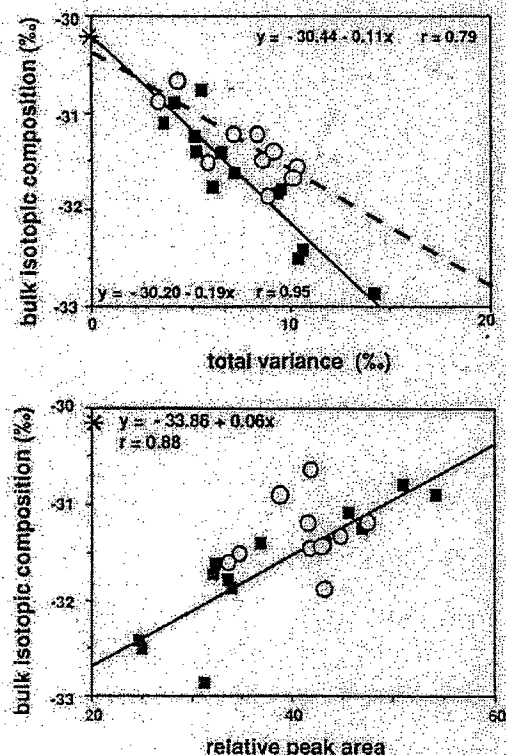


Fig. 8. a. Weighted mean isotopic composition of mixture B, calculated for repeated analysis of 10-ng (filled squares) and 70-ng (open circles) samples, plotted against total accuracy error for each run (solid line=10 ng, dashed line=70 ng; star indicates known isotopic composition). Furnace filling=0.35–1-mm-grade CuO.

b. Weighted mean bulk composition of mixture B plotted against total sample peak area for 10-ng (solid squares) and 70-ng (open circles) samples. (Regression line for 10-ng samples only; star indicates known composition.) Furnace filling=0.35–1-mm-grade CuO.

tion* (Fig. 8a) reveals that for both 10- and 70-ng sample sets the measured carbon isotopic composition is systematically depleted relative to the known isotopic composition calculated from determinations by conventional sealed-tube combustion. There is also an

*Bulk $\delta^{13}\text{C}_{\text{sample}} = m_n \delta^{13}\text{C}_{\text{alkane } n} + m_{n+1} \delta^{13}\text{C}_{\text{alkane } (n+1)} + \dots$ where m =proportion of total sample gas contributed by combustion of that compound; and n =number of carbon atoms in the lowest homologue in the series forming the standard mixture.

inverse linear correlation between the total variance and the weighted mean isotopic composition for both data sets. Furthermore, the range of total variance is reduced for the 70-ng compared to 10-ng samples. These results imply a link between sample size and accuracy. This is confirmed on cross-plotting the total sample peak area and the weighted mean bulk isotopic composition for each 10-ng analysis (Fig. 8b).

Despite attempts to inject consistently 10-ng quantities of sample, there is a two-fold variation in the amount of CO_2 analysed by the mass spectrometer (Fig. 8b). Additionally, injection of 70-ng samples is not reflected in a seven-fold increase in the amount of CO_2 analysed.

Variability in CO_2 abundance and $^{13}\text{C}/^{12}\text{C}$ on combustion of 10-ng samples (Fig. 8b) may be explained through incomplete mixing of CO_2 and He in the furnace tube. Gas is sampled at the open split, at a rate of $\sim 3 \text{ ml min}^{-1}$ (J. Jumeau, pers. commun., 1991), utilising the pressure drop, along the connecting capillary, to base pressure (10^{-9} Torr) in the analyser. Combustion of 70 ng of alkane must yield sufficient gas to saturate the He stream, effec-

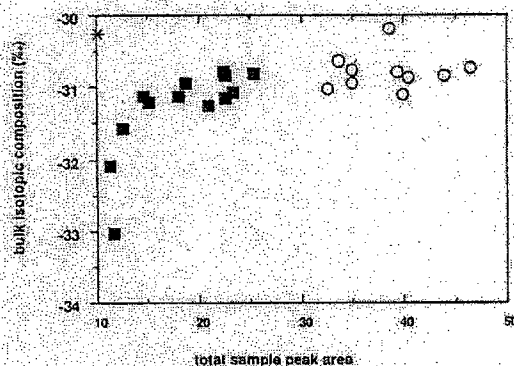


Fig. 9. Weighted mean isotopic composition of mixture B plotted against total sample peak area for 10-ng (solid squares) and 70-ng (open circles) samples. Above 20 area units there is no systematic variation in the bulk isotopic composition. Furnace packing=0.35–0.5-mm-grade CuO.

tively limiting the amount of CO_2 which can be sampled as a pulse passes through the furnace tube. In contrast, where the He stream is not saturated and homogeneous, selective uptake of $^{12}\text{CO}_2$, via a kinetic isotope effect, may generate erroneously low $\delta^{13}\text{C}$ -values. The latter effect may be more pronounced at low CO_2 abundances leading to the relationship evident in Fig. 8b.

The above experiment was repeated using a 0.65-mm-ID furnace tube filled with fine (0.35–0.5 mm grade) copper oxide. The results reveal a marked reduction in accuracy error and improved precisions (Table 1). Furthermore, for total peak areas > 20 area units, there is no interdependence between $^{13}\text{C}/^{12}\text{C}$ and CO_2 abundance (Fig. 9). Curtailed gas flow velocity and permeability through the finer packing must ensure a relative increase in the CO_2 abundance in the He stream and improved homogenization of the gas mixture.

From Fig. 8b it is evident that where isotopic fractionation at the open split is overcome (e.g., during analysis of 70-ng samples), the results are still ~1‰ lower than the known isotopic composition. In hindsight, we must conclude that during the period of this investigation the water trap in use was not effective, and resulted in measurement of low $^{13}\text{C}/^{12}\text{C}$ ratios as a result of the instrumental effects eluded to on p. 74.

Data acquired using a properly constructed water trap known to be working well (Table 1) shows further improvement in accuracy ($\leq \pm 0.63\text{‰}$) for 70-ng samples. There is a slight deterioration in precision although for all compounds, this remains better than $\pm 0.4\text{‰}$. Five determinations on mixture A (of lower-molecular-weight compounds) gave excellent precisions and accuracies ($\sigma_n \leq \pm 0.17\text{‰}$; $\Delta \leq \pm 0.15\text{‰}$). Evidently, there are other sources of instrumental error which affect higher-molecular-weight compounds. These sources of error, which are likely to be a function of boiling point, etc., are currently the subject of further investigation.

5. Conclusions

Measurements of reference gas $^{13}\text{C}/^{12}\text{C}$ ratios over a considerable range of major ion beam intensities (10^{-10} – 10^{-8} A) indicate that despite the continuous He flow to the mass spectrometer, there is no significant pressure effect resulting from peak broadening. This indicates that inaccuracy is unlikely to be introduced as a result of sample/reference imbalance.

From repeated analysis of standard mixtures we can also conclude that:

(1) There is a lower limit to sample size below which accuracy error increased dramatically. Errors approaching –3‰ have been induced by injection of small samples.

(2) A kinetic isotope effect at the open split may be responsible for much of the inaccuracy and poor precision, leading to erroneously negative $\delta^{13}\text{C}$ -values on sample peaks. This effect may be accentuated by use of coarse copper oxide furnace fillings which prevent complete mixing of the CO_2 -He stream. Fine copper oxide (0.35–0.5 mm grade) may promote gas mixing and decrease the kinetic isotope effect at the open split, resulting in improved accuracies and precisions.

(3) Water released from the cryogenic trap either through overnight defrosting, or through cold-trap inefficiency can also lead to erroneously negative sample values. This effect can be overcome by nightly baking of the cold trap, and strict adherence to the water trap structure currently recommended by the manufacturer.

(4) Analyses obtained at the optimum conditions determined in this study: (a) furnace tube packing = 0.35–0.5 mm grade, with (b) a well-maintained water trap gave precisions generally better than $\pm 0.4\text{‰}$ and accuracies better than $\pm 0.65\text{‰}$ on high-molecular-weight standards. Low-molecular-weight compounds gave excellent data (both precision and accuracy better than $\pm 0.2\text{‰}$). Further work is evidently required to determine sources of error

specific to higher-molecular-weight compounds.

Acknowledgements

We are indebted to J. Jumeau, P. Freedman and J.E. Rouse for invaluable discussions during the course of this investigation and Professor C.T. Pillinger for comments on a previous draft. Dr. I. Franchi supplied standard mixture B. SURRC is funded by NERC and the Scottish Universities.

References

- Craig, H., 1957. Isotopic standards for carbon and oxygen and correction factors for mass spectrometric analysis of carbon dioxide. *Geochim. Cosmochim. Acta*, 12: 133-149.
- Fallick, A.E. and Baxter, M.S., 1977. The pressure effect and peak broadening in gas source stable isotope mass spectrometry. *Int. J. Mass Spectrom. Ion Phys.*, 25: 155-165.
- Mathews, D.E. and Hayes, J.M., 1978. Isotope-ratio-monitoring-Mass spectrometry. *Anal. Chem.*, 50: 1465-1473.
- Mook, W.G. and Grootes, P.M., 1973. The measuring procedure and corrections for the high precision mass spectrometric analysis of isotopic abundance ratios, especially referring to carbon, oxygen and nitrogen. *Int. J. Mass Spectrom. Ion Phys.*, 12: 273-289.
- Sano, M., Yotsui, Y., Abe, H. and Sasaki, S., 1976. A new technique for the detection of metabolites labelled by the isotope ^{13}C using mass fragmentography. *Biomed. Mass Spectrom.*, 3: 1-3.
- Craig, H., 1957. Isotopic standards for carbon and oxygen and correction factors for mass spectrometric analysis

REVIEW

Referencing strategies and techniques in stable isotope ratio analysis

Roland A. Werner and Willi A. Brand*

Max-Planck-Institute for Biogeochemistry, P.O. Box 100164, 07701 Jena, Germany

Received 5 December 2000; Revised 6 December 2000; Accepted 23 December 2000

SPONSOR REFEREE: T. B. Coplen, US Geological Survey, 431 National Center, Reston, VA 20192, USA

Stable isotope ratios are reported in the literature in terms of a deviation from an international standard (δ -values). The referencing procedures, however, differ from instrument to instrument and are not consistent between measurement facilities. This paper reviews an attempt to unify the strategy for referencing isotopic measurements. In particular, emphasis is given to the importance of identical treatment of sample and reference material ('IT principle'), which should guide all isotope ratio determinations and evaluations. The implementation of the principle in our laboratory, the monitoring of our measurement quality, the status of the international scales and reference materials and necessary correction procedures are discussed. Copyright © 2001 John Wiley & Sons, Ltd.

Roughly 15 years ago a new and relatively economic chromatographic method to measure stable isotope ratios with high precision became commercially available. Carrier gas or on-line isotope ratio analysis systems now cover a wide range of applications and have led to a dramatic increase in the number of stable isotope ratio measurements worldwide. Although this has been a benefit to this field of science as a whole, it has also created a number of problems. We feel that the depth of knowledge that the few original experts possessed has been diluted considerably over the years with the consequence that the overall precision and reliability of isotope ratio values may have declined. In particular, the accuracy of reported δ -values relative to an international standard may have suffered from the vast increase in analyses made.

On the other hand, such a large increase in the number of analyses offers the opportunity to establish fully automated analysis sequences that include a proper referencing strategy. This opportunity should enable the reliability of isotope ratio value assignment to be improved. Although this has happened to a certain extent in many laboratories, referencing strategies have certainly not been unified throughout the community.

This review will be of considerable value both in

educating students and also newcomers to the exciting field of high precision stable isotope ratio measurements. In particular, we want to emphasize techniques for reliable standardization in carrier gas or on-line techniques where an accepted protocol for assigning δ -values^a on an internationally accepted scale has not yet been defined. We feel particularly suited for this task because we were given the opportunity to start a large isotope ratio measurement facility from scratch two years ago and thus had to develop all the techniques and protocols for such an endeavor recently.

Stable isotope ratios and their international scales

The stable isotope ratios measured most widely include $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$, $^{18}\text{O}/^{16}\text{O}$ and $^2\text{H}/^1\text{H}$ (or simply D/H). Other less frequently measured ratios are inter alia $^{17}\text{O}/^{16}\text{O}$, $^{34}\text{S}/^{32}\text{S}$, $^{33}\text{S}/^{32}\text{S}$. The common feature of these ratios is that they can be determined using a few light gases (CO_2 , CO , N_2 , O_2 , H_2 , and SO_2). Hence they share a common technology termed (Gas) Isotope Ratio Mass Spectrometry.

The principle of the classical method is very easy to understand: Two gases are stored in containers connected via capillaries to a switching unit, the 'changeover valve'¹ (Fig. 1). The latter serves to direct one of the gases to the ion source of an isotope ratio mass spectrometer (IRMS)^{2,3,4,5,6} while the other gas flows to a waste vacuum line, and vice versa. The ion currents are measured separately from both gases and compared a number of times. The measured relative difference in ion current ratios is then calculated relative to an internationally agreed isotope ratio scale.

Table 1 is a compilation of the international stable isotope ratio scales in use together with the presently accepted absolute ratios and their errors. When comparing samples

*Correspondence to: W. A. Brand, Max-Planck-Institute for Biogeochemistry, P.O. Box 100164, 07701 Jena, Germany.
E-mail: wbrand@bgc-jena.mpg.de
E-mail: rwerner@bgc-jena.mpg.de

^a The variation of stable isotope ratios in nature is small. The small differences are conveniently expressed as delta values (δ) in per mill [‰] deviation from a reference^{2,3,8} according to

$$\delta[\text{‰}] = (R_{\text{sa}}/R_{\text{ref}} - 1) \cdot 1000 \quad (1)$$

R_{sa} and R_{ref} are the sample and reference isotope ratios, respectively.

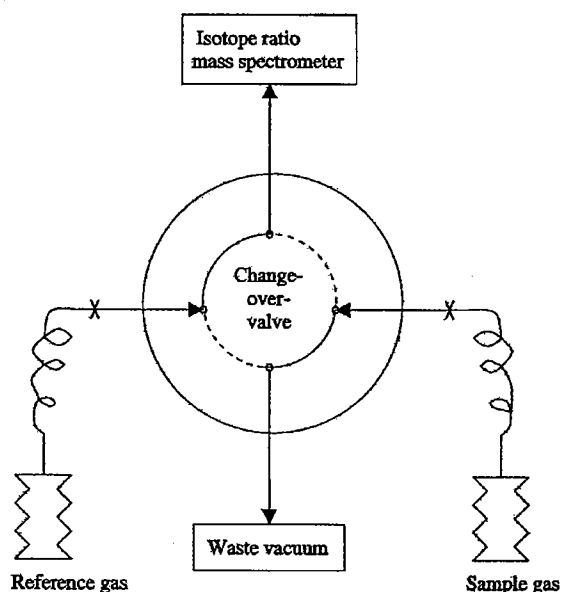


Figure 1. Schematic representation of a dual inlet system featuring the 'Changeover Valve' as the classical referencing technique in Stable Isotope Ratio Mass Spectrometry. While the gas in one volume is flowing to the mass spectrometer, the gas in other goes to a waste line and vice versa.

with one of the standard materials, however, the errors are generally considerably smaller owing to the fact that a relative measurement has far fewer sources of error than an absolute determination.

Carbon

The international scale for $^{13}\text{C}/^{12}\text{C}$ started as a carbonate laboratory standard for oxygen and carbon isotope ratios in the group of Harold Urey at the University of Chicago in the early 1950s.^{7,8} Being representative for carbon in the lithosphere that precipitated from the world oceans, PDB was later proposed as the international reference material for the carbon and oxygen δ -scale by Harmon Craig.⁹ The Cretac-

eous belemnite material was picked from the Pee Dee formation in South Carolina, hence 'PDB' for Pee Dee Belemnite. The original material no longer exists. It has been replaced by assigning exact δ -values (both $\delta^{13}\text{C}$ and $\delta^{18}\text{O}^b$) to another carbonate (NBS-19) relative to PDB.^{10,11} This new scale is termed 'VPDB' (Vienna PDB) in recognition of the role that the International Atomic Energy Agency (IAEA), located in Vienna, has played in redefining the PDB scale. The IAEA played a similar role for the other δ -scales that have a 'V' preceding the original scale name.

Unfortunately, isotope ratios of CO_2 must be determined using the molecular ion masses 44, 45 and 46. The ^{13}C information comes in disguise. It is available from the 45/44 ion current only after subtracting the $\sim 7\%$ contribution from $^{12}\text{C}^{17}\text{O}^{16}\text{O}^+$ (see 'Correction of isobaric interferences'). This complicates the exact comparability of $\delta^{13}\text{C}$ values between different laboratories.

Oxygen

High precision isotope ratio measurements of oxygen were first made on CaCO_3 , O_2 and H_2O . Again it was Urey's group that developed these applications for paleoclimate reconstruction.^{2,19,20,21} The two accepted international scales (VSMOW and VPDB) have developed over time. A variety of carbonate (including PDB) and water standards were used until 1961, when Harmon Craig proposed SMOW (Standard Mean Ocean Water). SMOW isotopically represents the hydrosphere with the world oceans as the largest reservoir of oxygen (and hydrogen). Interestingly, the standard that Craig proposed was not available as a reference material. It had a δ -value that was believed to represent average ocean water based on experience accumulated over time. It was precisely defined relative to the (now exhausted) water standard NBS-1 that was almost 8‰ off the new $\delta^{18}\text{O}$ -scale and 47.6‰ off the simultaneously defined δD -scale. Both Craig and the IAEA engaged in developing a reference material with an isotopic composition of SMOW, which led to the current VSMOW scale. This material presently is still available in limited amounts from the IAEA in Vienna. A new batch of reference water matching the VSMOW scale as closely as possible in all isotope ratios is currently being prepared.

$^{18}\text{O}/^{16}\text{O}$ ratios are measured using CO_2 after appropriate conversion from the original material. Due to the fact that the

^b $\delta^{18}\text{O}_{\text{NBS-19}} \equiv -2.2\text{‰}$ vs. VPDB; $\delta^{13}\text{C}_{\text{NBS-19}} \equiv +1.95\text{‰}$ vs. VPDB

Table 1. International isotope ratio scales¹²

| Isotope ratio | International scale | Accepted ratio [$\times 10^6$] | Error of ratio | Error of ratio [%] | Ref. |
|-------------------------------|---------------------|----------------------------------|----------------|--------------------|--------------------------------------|
| $^{13}\text{C}/^{12}\text{C}$ | VPDB | 11180.2 ^c | ± 2.8 | ± 2.5 | Chang and Li ¹³ |
| $^{18}\text{O}/^{16}\text{O}$ | VSMOW | 2005.2 | ± 0.45 | ± 0.22 | Baertschi ¹⁴ |
| | VPDB | 2067.2 ¹² | | | using +30.92‰ |
| $^{17}\text{O}/^{16}\text{O}$ | VSMOW | 379.9 | ± 0.8 | ± 2.11 | Li <i>et al.</i> ¹⁵ |
| | VPDB | 386.0 ¹² | | | with +30.92‰ and $\lambda = 0.52$ |
| $^{15}\text{N}/^{14}\text{N}$ | AIR-N ₂ | 3678.2 | ± 1.5 | ± 0.41 | DeBievre <i>et al.</i> ¹⁶ |
| $^2\text{H}/^1\text{H}$ | VSMOW | 155.75 | ± 0.08 | ± 0.51 | De Wit <i>et al.</i> ¹⁷ |
| $^{34}\text{S}/^{32}\text{S}$ | VCDT | 44150.9 | ± 11.7 | ± 0.27 | Ding <i>et al.</i> ¹⁸ |

Isotope ratios are reported as δ -values in per mill (‰) deviations from the origin of the respective international scale. Please note that for oxygen two internationally accepted scales coexist.

^c Chang and Li report a $^{13}\text{C}/^{12}\text{C}$ value of 0.011202 for NBS-19. The $\delta^{13}\text{C}$ -value of NBS-19 is +1.95‰, defining the VPDB scale. The previously established $^{13}\text{C}/^{12}\text{C}$ PDB ratio value⁹ of 0.0112372 is 5‰ higher.

behavior of terrestrial ^{17}O and ^{18}O are closely linked via mass fractionation laws, $^{17}\text{O}/^{16}\text{O}$ ratios (measured using O_2 gas) are analyzed only occasionally.

When carbonates or CO_2 in air are measured, oxygen isotope ratio values are preferentially reported on the VPDB or VPDB_{gas} scale and this can lead to confusion when the scales are not stated explicitly. The reason for the coexistence of two oxygen scales is that measurements of δ -values against a chemically identical or similar reference can in principle be made with enhanced precision. VPDB has a $\delta^{18}\text{O}$ value of +30.92‰²² on the VSMOW scale. The CO_2 gas 'developed' from hypothetical VPDB (= VPDB_{gas}) at 25°C using pure, water-free H_3PO_4 is 10.25‰ heavier;^{23,d} it has a $\delta^{18}\text{O}$ value of +41.5‰ on the VSMOW scale. It should be noted that δ -values can not be added or subtracted in a simple way when referring to different standards.^e

Hydrogen

The isotopes of hydrogen have the largest relative mass difference. Since most isotope fractionations in nature roughly scale with relative mass difference, the largest variations in terrestrial isotope ratios are found for hydrogen. Although the large mass difference makes the isotope signatures easier to detect, the low abundance of deuterium (only about 150 ppm in ocean water) can create problems during high-precision analysis. Consequently, two international reference materials are in frequent use to cover the natural range of isotope ratios for D/H. The basis of the international scale is the same as oxygen, VSMOW. The other reference material is a water sample named SLAP (Standard Light Antarctic Precipitation) that is, relative to VSMOW, more than 42% depleted in deuterium (−428‰). The difference between VSMOW and SLAP is used to correct for the nonlinear behavior of instrumentation and (or) sample preparation during analysis.

Nitrogen

Unlike the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values in atmospheric CO_2 , the $\delta^{15}\text{N}$ value of atmospheric N_2 does not change within measurement precision over time or space.^{24,25} This is due to the fact that the atmospheric pool of nitrogen is by far the largest of all nitrogen-bearing pools on earth and thus cannot be altered significantly by known processes. It participates in all natural processes as source and sink so that the net change over time is close to zero. Consequently, AIR- N_2 has been adopted as the international reference material for all nitrogen isotope ratio analyses. This does not imply that the isotope ratios are easy to measure. From air, N_2 must be isolated without isotopic fractionation for use as a primary reference. For convenience (and thus enable better data comparability) the IAEA has made secondary standards (mostly ammonium and nitrate salts) available for direct use in sample preparation lines involving combustion.

^d More recently, a value of 10.44‰ has been reported by Kim and O'Neil.⁵²

^e The δ -value for a sample (sa) measured against a working standard (ws) on an international scale (is) is given by

$$\delta_{\text{sa/is}} = \delta_{\text{sa/ws}} + \delta_{\text{ws/is}} + \delta_{\text{sa/ws}} \times \delta_{\text{ws/is}}/1000 \quad (2)$$

Sulfur

Sulfur is most commonly measured as SO_2 gas on the molecular ion masses 64 and 66. Unfortunately, the isotopes of oxygen directly interfere with the sulfur isotopes (isobaric interferences) at these mass positions and thus cannot be measured independently (the ion current at m/z 66 comprises $^{34}\text{S}^{16}\text{O}_2^+$ and $^{32}\text{S}^{16}\text{O}^{18}\text{O}^+$). Using SF_6 as the measurement gas avoids this problem, but preparation of SF_6 requires fluorine gas as a reagent and preparing clean SF_6 is difficult. Hence, SF_6 has found only limited use. The international δ -scale is defined by VCDT (Vienna Cañon Diablo Troilite)²⁶ The original CDT,²⁸ adopted as the primary standard in 1960, was prepared from the FeS phase of a large iron meteorite found at Meteor Crater, Arizona. Improved measurement precision has revealed that CDT is not sufficiently homogeneous²⁷ to be continued as the primary reference material and, as a consequence, the CDT scale has been replaced with VCDT. VCDT is defined by assigning a $\delta^{34}\text{S}$ -value of −0.3‰ (exactly) to IAEA-S-1 (formerly NZ-1, Ag_2S).²⁶ It is worth noting that the absolute $^{34}\text{S}/^{32}\text{S}$ ratio in VCDT given in Table 1 differs from the previously established value (45004.5)²⁸ for CDT by about −20‰. This affects the correction for ^{18}O contribution (see 'Correction of isobaric interferences').

Referencing techniques in isotope ratio mass spectrometry and the principle of identical treatment

Dual inlet system and changeover valve. The breakthrough in classical isotope ratio mass spectrometry was the dual inlet mass spectrometer, introduced by Urey in 1948² and described by McKinney *et al.* in 1950.³ Despite significant improvements in electronics, vacuum system design and full computerization, the basic principles embodied in this mass spectrometer still form the basis of modern stable isotope ratio mass spectrometers for high-precision analysis of clean analyte gas.

A key feature of the McKinney instrument is the 'changeover valve'¹ (Fig. 1), which allows continuous flows of reference and sample gas to be alternated between the mass spectrometer and a waste line vacuum pump. The 'changeover valve' is the classical referencing technique. Together with the dual variable volume (or mercury piston) reservoir system for introduction of sample and reference gases, it allows a direct comparison of ion currents and ion current ratios. Instrumental effects like temporal fluctuation of sensitivity or temperature drifts cancel almost completely.

In order to avoid isotopic composition changes of the gas in either reservoir over time due to molecular effusion into vacuum, the connection between the gas reservoirs and the 'changeover valve' is made with thin capillaries (0.1–0.2 mm i.d.) of about 100 cm length that are crimped near the end.²⁹ The hole right at the capillary crimp also constitutes a molecular leak. However, provided the pressure in the capillary is sufficiently high (typically >50 mbar for hydrogen and >15 mbar for other gases), the flow through the capillaries is viscous, not molecular, and thus prevents the isotopically enriched gas at the crimp from diffusing back into the reservoirs.⁴ This viscous flow condition establishes a

Table 2. Measurement of two CO₂ gas samples by different laboratories

| $\delta^{13}\text{C}$ [‰ VPDB] | | | | | | | |
|---|--------|---------|--------|------------|---------|--------|-----------|
| Sample | CIO | INSTAAR | CSIRO | Heidelberg | Scripps | mean | std. dev. |
| GS-19 | -7.502 | -7.517 | -7.516 | -7.539 | -7.465 | -7.508 | 0.0273 |
| GS-20 | -8.622 | -8.624 | -8.617 | -8.635 | -8.575 | -8.615 | 0.0231 |
| $\delta^{18}\text{O}$ [‰ VPDB-CO ₂] | | | | | | | |
| Sample | CIO | INSTAAR | CSIRO | Heidelberg | Scripps | mean | std. dev. |
| GS-19 | -0.193 | -0.790 | -0.617 | -0.426 | -0.126 | -0.430 | 0.280 |
| GS-20 | -0.991 | -1.580 | -1.380 | -1.191 | -0.915 | -1.211 | 0.274 |

lower limit to the minimum amount of gas that can be determined with high precision. Small amounts of gas may be frozen into a small volume ('microvolume') in front of the capillary. Making this reservoir as small as is physically possible (~250 μL) and operating at 15 mbar results in a minimum sample size of about 4 μL^{f} gas.

Using modern dual inlet mass spectrometers it is possible to obtain a precision of 0.01‰ for $\delta^{13}\text{C}$ (and similar values for other isotope ratios) by comparing the ion current ratios of the gases in both reservoirs a number of times for several seconds. The mean is calculated and such measurements are compared a number of times during the course of a day. However, precision is not the same as accuracy. On the next day the results may again be very precise. The mean, however, may differ from the mean from the previous day, or from the previous week, month, or year.

The situation is illustrated by results from a recent intercomparison of CO₂ gas initiated by the Groningen Centre for Isotope Research (CIO).³⁰ The ring test involved two gases (GS-19 and GS-20) sent to five prominent laboratories for isotopic determination. The results from the ring test are given in Table 2.

The precision of the $\delta^{13}\text{C}$ -values is approx. 0.025‰ and appears acceptable, whereas the $\delta^{18}\text{O}$ data show an unacceptably large scatter. This finding is attributed to different referencing methods or materials in the laboratories. If GS-19 is used as the reference (δ -value set to 0.0‰) and GS-20 measured against it, the precision of the reported differences improves to 0.009‰ for $\delta^{13}\text{C}$ and 0.016‰ for $\delta^{18}\text{O}$. Through direct referencing, the $\delta^{13}\text{C}$ results are improved by a factor of 3 and the $\delta^{18}\text{O}$ situation improves almost 20-fold.

How can the precision that these mass spectrometers are obviously able to deliver be conserved and converted into an accuracy figure over long time periods? How can different mass spectrometers deliver results that can be compared reliably at a precision level of 0.01‰?

These questions require some discussion before a more

general conclusion can be reached. Several points need to be considered:

- The capillaries to the 'changeover valve' are carefully heated and the inlet system is conditioned until the ratios for the same gas, introduced to both reservoirs, is close to zero ('zero enrichment test').⁸ However, in the real world, the two gases are different and may even differ in their trace gas impurities. Traces of water or other protonation agents can give rise to an isobaric interference of CO₂H⁺ (m/z 45) resulting from ion/molecule reactions in the ion source. Similar protonation reactions are possible for other gases resulting in H₃⁺ or N₂H⁺.
- The isotopic composition of a sample and reference gas should not be very different; however, in reality, they always will be and, as a consequence, balancing the major ion beam means that the minor beams will have different readings. If the response of the measurement channels is not perfectly linear (and it never will be), this is a source of error that can vary with time.
- The measurement precision and results for $\delta^{18}\text{O}$ from CO₂ gases depend critically on the nature and the cleanliness of the internal surfaces of the reservoir and the transfer capillaries. Not only can CO₂ exchange oxygen with surface water, it may even exchange with other forms of loosely bound oxygen on the surface and hence cause uncontrolled isotopic fractionation. This may be different for the two capillaries and may change over longer time periods.
- Different mass spectrometers (even from the same manufacturer) can have several sources of error in determining a given isotopic difference. This has been shown for hydrogen where the isotope ratio differences are often large. It is also important for other gases like CO₂ when the measured δ -values differ by 10‰ or more and the required accuracy is high.

It is common practice in laboratories that strive for the highest long-term precision and accuracy to have sample and reference gas measured from the same (sample) reservoir of the dual inlet system. The gas in the reference volume serves as a temporary mediator between the different runs. Proceeding in this manner, sample and reference gas are handled in a highly comparable, almost equal, fashion and, as a result, some (but not all) of the errors discussed above tend to cancel out.

^f 1 μL ('bar microliter') is an amount of 1 μL gas at STP.

⁸ Please note that crimping of the capillaries will not necessarily result in the same signal height for the same gas pressure on both sides. In fact, when referencing through the sample line ('IT principle'), it is more important to have a long lifetime for the standard gas and thus make the crimp for the capillary tighter on this side.

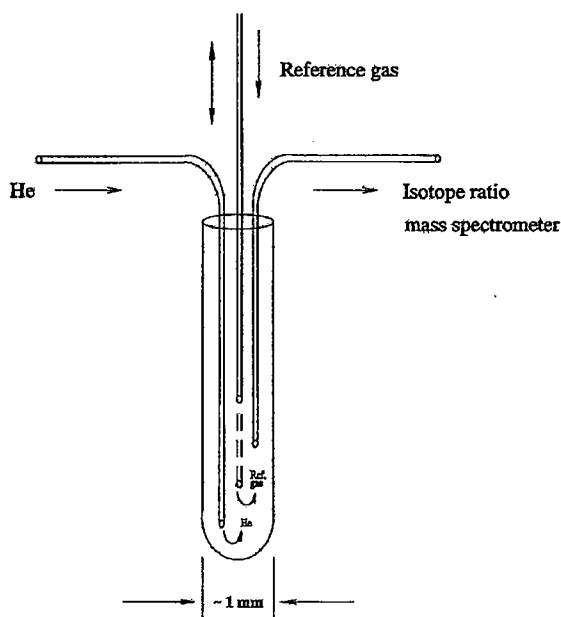


Figure 2. Referencing technique in isotope ratio monitoring applications. Helium enters a small glass tube through a fused-silica capillary. Another fused-silica 'sniffing' capillary (usually 1000 mm long and 0.1 mm i.d.) connects to the ion source of the mass spectrometer. Reference gas pulses are generated by moving a third capillary between two positions, one upstream and the other downstream from the sniffing point.

The IT principle

We will refer to 'Identical Treatment' of sample and reference material as the 'IT principle' for relative measurements. The IT principle, which does not apply exclusively to the dual inlet technique, serves excellently for maintaining long-term laboratory performance records for other isotope ratio measurement techniques as well (see 'Performance charts').

It is rare that the measurement gas is identical to the original sample. Typically, a carbonate, a water sample, an organic compound, a piece of bone, soil, hair or a gas mixture etc., is selected from which the measurement gas has to be produced or extracted. During this transformation it is critical to avoid isotopic alteration. In carbonate analysis or water equilibration the IT principle has been common practice for a long time. Carbonates are effectively standardized by running carbonate reference material alongside the samples on a daily routine basis. Water samples are equilibrated together with reference water standards in the same temperature-controlled bath. Because the isotopic fractionation occurring in the CO_2 liberation or the isotope transfer reaction to the gas phase is strongly temperature dependent, the reference material is subjected to the same reaction conditions. The isotopic fractionation cancels as long as the chemical nature of sample and reference is closely comparable. The same argument applies to water reduction methods for hydrogen isotope ratio analysis. Here, irrespective of the specifics of the reduction process, the

potential for isotopic fractionation is encountered in almost every detail of the reaction including the type of vessel, the temperature, the amount of reduction material, and the timing sequence of the reduction reaction. Hence, running reference water samples concurrently with unknown samples in every batch is required in order to obtain reliable results.

Isotope ratio monitoring techniques

Isotope ratio monitoring ('irm') encompasses all techniques for high-precision determination of stable isotope ratios that employ a helium carrier gas for transferring the analyte gas as a transient into the isotope ratio mass spectrometer.³¹ The term 'irm-GC/MS' was introduced by D.E. Matthews and J.H. Hayes in their pioneering paper in 1978.³² Popular acronyms in common use for specific irm couplings are GC/C/IRMS (GC/combustion/IRMS) and EA/IRMS (elemental analyzer/IRMS). The techniques were originally developed to measure $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$. More recently, D/H, $^{34}\text{S}/^{32}\text{S}$ and $^{18}\text{O}/^{16}\text{O}$ ratio determination has become available, accompanied by a host of new acronyms.

In irm systems the sample gas leaves the appropriate sample preparation and separation device as a peak entrained in helium. The chromatographic separation not only ensures that the analyte gas is pure or coming from a single precursor, it also separates the stable isotopes (i.e. isotopomeric molecules) of the analyte to a certain extent. In irm-GC/MS applications the separation often exceeds 100 ms between e.g. the ^{13}C moiety of the analyte, which elutes first, and its ^{12}C counterpart.^{31,33}

Referencing can be carried out in a number of ways. The most common method is to inject pulses of reference gas directly into or in parallel to the GC effluent stream.³⁴ The time window or windows for the reference gas pulses must be carefully selected in order to avoid interference with any analyte material entering the mass spectrometer concurrently. Other methods include the admixture of reference compounds to a GC mixture (internal standards) or, less favorably, the direct comparison of ratios from chromatogram to chromatogram.

Figure 2 shows schematically an example of a reference gas injection mechanism. Inside a closed, thin (<1 mm) glass tube, the effluent stream from the interface is fed in continuously with a fused-silica capillary. Downstream a second fused-silica capillary acts as the transfer line to the mass spectrometer. Usually, this capillary has an internal diameter of 0.1 mm and a length of 1000 mm. Together with the pressure drop from atmosphere to about 10^{-5} mbar, this capillary allows for a flow of about 400 μL per minute. The flow through long, thin capillaries under viscous flow conditions follows the law of Hagen-Poiseuille. For a given pressure difference the flow is linked to the 4th power of the radius and linearly to the length of the capillary. Hence, when doubling the radius, the flow will increase by a factor of 16 while keeping the other parameters constant. The third capillary (see Fig. 2) serves to inject pulses of reference gas. Using a piston mechanism it can be switched between two positions: one upstream and one downstream from the sniffing point of the transfer capillary. Only the upstream position will deliver reference gas to the sniffing point.

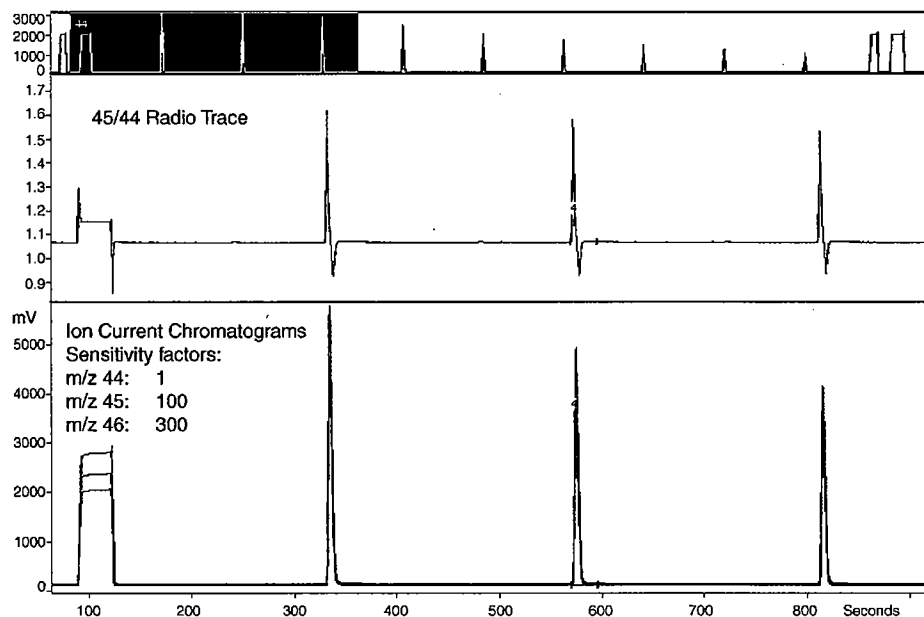


Figure 3. Graphical output of a irm-GC/MS system, in this case from a Gasbench II run (see $\delta^{18}\text{O}$ determination). Three GC traces are monitored simultaneously with different sensitivity factors. The upper trace is an overview over the whole run. The middle window ('45/44 Ratio Trace') represents the instantaneous 45/44 ion current ratio. The first, flat-topped peak represents a post column injection of reference gas. For the sample peaks, please note the isotope swing going positive first which is indicative of the partial separation of the isotopomers on the GC column. Reference pulses do not show this behavior. At the flanks they exhibit a reaction to the fast changing intensity due to differences in the amplifier time constants.

Downstream the helium carrier will sweep away any effluent from capillary three. Figure 3 shows an example of a simple GC run (in $\delta^{13}\text{C}$ measurement mode). The first, flat-topped peak is a reference gas injection lasting for about 30 s. The other peaks are from sample gas analyses. Their shape is typical for this type of gas chromatography. Please note the variation of the instantaneous (45/44)-ratio along each GC peak going positive first due to the ^{13}C moiety eluting earlier than the pure ^{12}C compound.

The principle of moving capillaries in the open split region has been utilized in a number of ways. Multiple reference gases can be introduced, the GC effluent gas can be diluted with helium further to cover a wider dynamic range³⁵ or the transfer capillary may be switched into a clean helium cushion for 'heartcutting' (i.e. selecting or selectively discarding) GC peaks.

Other means of injecting reference gas pulses include rotary valves equipped with an injection loop or carefully designed multiple valve systems. The drawback common to all of these systems is that during switching a short interruption of the continuous flow of carrier gas occurs which leads to problems in the background definition of the reference gas peaks and, hence, to reduced precision.

It is important to note that referencing solely relative to reference gas pulses is an immediate violation of the IT principle introduced above. Sample peaks are generated from original material going through a combustion/separation or separation/combustion step whereas the (working)

reference is pure gas. The principle governing long-term precision and, more importantly, accuracy requires that sample and reference be identical in nature and follow the same sample isolation and conversion pathway. Hence, a *n*-heptadecane sample should be referenced with a *n*-heptadecane reference.

Often gas chromatograms are very complex. The conversion efficiency of *n*-heptadecane should be very close to *n*-hexadecane or *n*-octadecane. Even an *n*-C25 alkane should be a valid reference point provided the stability of the mass spectrometer is high and the conversion unit reliably works close to 100% yield. Hence, even whole chromatograms can serve as references in pursuit of the IT principle. In this case, reference gas pulses again take the role of a mere mediator between runs, very much as the standard gas in dual inlet analyses. In principle, the number of reference runs is determined by the precision required for the experiment in question.

The workhorse in most modern isotope ratio laboratories is the combination of an elemental analyzer with isotope ratio mass spectrometry (EA-IRMS or irm-EAMS, sometimes termed BSIA⁴ for bulk sample isotope analysis). As with irm-GC/MS, the immediate or working referencing is commonly made through co-injected reference gas pulses into the effluent stream. Earlier commercial versions of this instrumentation relied solely on comparing from sample to sample, without reference gas peaks. However, precision of a single measurement is improved when comparing

standard and reference more closely in time. Data security is an issue when a single result relies on the measurement of the whole sequence.

In order to work under the rule of the IT principle individual sample results versus a gaseous working standard can be compared with results from reference material that has passed the full sample line in the same sequence of measurements.

Day-to-day working environment for high-precision isotope ratio analyses and generation of performance charts

In our laboratory we have implemented a number of routine measurement schemes for the following types of analyses:

- $\delta^{13}\text{C}$ from bulk solid (and liquid) material (irm-EAMS system)
- $\delta^{15}\text{N}$ from bulk solid (and liquid) material (irm-EAMS system)
- δD from water samples (Cr-reduction/dual inlet)
- $\delta^{18}\text{O}$ from water samples ($\text{CO}_2/\text{H}_2\text{O}$ equilibration followed by irm analysis)
- $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ from CO_2 in air samples (cryogenic separation/dual inlet, in development)
- O_2/N_2 ratios in air samples (changeover system with open split, in development)

The first step in setting up routine protocols is the establishment of effective laboratory standards that can be used and monitored on a daily basis. General rules for laboratory standards are difficult to fix, they largely depend on the type of analysis.

The second step is the development of consistent analysis sequences, i.e. the decision how on a daily basis samples, reference material, and blanks (if applicable) are positioned in a sample carousel or autosampler. In this respect, economic aspects, such as the number of samples per day and week, scheduling of routine maintenance, number of samples per reactor, etc., are of importance and must be considered with great care and separately for every type of analysis. In our laboratory we have tried to adjust the measurement cycles to the diurnal and weekly cycle of the human operators, to optimize throughput without sacrificing precision.

Selection of laboratory reference materials

After having obtained the relevant primary reference material from the IAEA or other agency, secondary or laboratory standards must be prepared for every day use. The IAEA recommends not using the original material on a

daily basis. Primary reference materials in general are in short supply and their commercial availability is restricted.

For $\delta^{13}\text{C}$ analysis using a combustion technique, NBS-22 (-29.78‰ ^h vs. VPDB) is a valid reference material for almost any purpose. The secondary laboratory standard should be chosen according to the following criteria:

- easy to handle during weighing or other preparatory steps.
- preferably a pure single chemical compound.
- isotopically homogenous down to the smallest amount used during analysis.
- stable and constant in isotopic content over a long time period. For liquids, in particular water, large quantities are advantageous in order to minimize evaporation losses. The isotopic composition should be stable versus frequent use.ⁱ
- easy to replace if exhausted.
- chemically identical or close to the samples to be measured. This point must be evaluated in terms of the corresponding analysis. It is probably easy to use some oily material like NBS-22 for referencing almost any bulk combustion ($\delta^{13}\text{C}$). However, using carbonates for referencing water samples is more difficult if not impossible in pyrolysis systems ($\delta^{18}\text{O}$).³⁶
- the isotopic composition should be in the range of the samples to be measured. Isotopic differences can be measured most precisely when small. Because most natural organic material is produced primarily through C_3 -photosynthesis, $\delta^{13}\text{C}$ values around -25‰ vs. VPDB are recommended for the laboratory standard.
- non-hygroscopic. This is of particular importance for oxygen and hydrogen isotope ratio analysis using high-temperature carbon reduction ('pyrolysis') systems.

irm-EAMS

We analyze any bulk material that can be combusted quantitatively to CO_2 , N_2 and H_2O (+ other oxides if applicable) using the combination of an elemental analyzer (NA 1110, CE Instruments, Milan, Italy) with an isotope ratio mass spectrometer (DELTA⁺XL, Finnigan MAT, Bremen, Germany). The coupling interface is a ConFlo II[®], modified in-house to a ConFlo III,³⁵ which includes two open splits, one for the coupling, the other one for reference gas introduction. The coupling split can be varied over a wide range from zero to 64-fold dilution of the effluent stream without causing detectable isotopic fractionation. Two reference gases (mostly CO_2 and N_2 , alternatively H_2 and CO) can be actuated under computer control. H_2 and CO reference gases are used when the system operates in high-temperature 'pyrolysis' mode with a Finnigan MAT TC/EA for D/H or $^{18}\text{O}/^{16}\text{O}$ isotope ratio analysis. The operation of the system generally follows a well-defined protocol. Samples, reference material aliquots, and blanks are weighed and filled into the 32-position autosampler carousel according to a loading list (Fig. 4). Please note that each list represents samples for three carousels (we use 96-position sample trays for storing samples in a desiccator before measurement). It serves as input for the sequence information in the mass spectrometer control software.

The prefilled positions in the loading list are mandatory. They are used in a post-run off-line evaluation on a

^h The previously recommended value of -29.74‰ was adjusted to -29.78‰ by the 8th Advisory Group Meeting on Future Trends in Stable Isotope Reference Materials and Laboratory Quality Assurance, IAEA, Vienna, Sept. 18–22, 2000.

ⁱ Carbonates can exchange oxygen isotopes with air- CO_2 . This effect depends on grain size, humidity, nature of the carbonate, and frequency of opening when the material is kept under inert gas. Water reference material can alter its isotopic composition through evaporation and transpiration through container walls. Hence, the frequency of opening the reference water container can affect the isotopic stability. GC injection mixtures may change their isotopic composition and concentration via evaporation when low boiling point material is used as a reference.

EA Loading List - C only

Total weight max.: 100 mg Sample, without Dil.: 30-210µg C, plus Dil.: max. 5000 µg C

Name: Date: C%: Tray identity:

| | Line | | Sample | [mg]Total Weight | | Sample | [mg]Total Weight | Line | |
|--|------|-----|------------------------|---------------------|-----|--------------|---------------------|------|--|
| | 1 | A1 | Ali-j1 | | E1 | | | 49 | |
| | 2 | A2 | bl (blank) | | E2 | | | 50 | |
| | 3 | A3 | Ali-j1 | | E3 | | | 51 | |
| | 4 | A4 | Ali-j1 [Ref] (71,09%C) | | E4 | | | 52 | |
| | 5 | A5 | | | E5 | | | 53 | |
| | 6 | A6 | | | E6 | | | 54 | |
| | 7 | A7 | | | E7 | | | 55 | |
| | 8 | A8 | | | E8 | | | 56 | |
| | 9 | A9 | | | E9 | | | 57 | |
| | 10 | A10 | | | E10 | | | 58 | |
| | 11 | A11 | | | E11 | | | 59 | |
| | 12 | A12 | Ali-j1 | | E12 | | | 60 | |
| | 13 | B1 | bl | | F1 | | | 61 | |
| | 14 | B2 | bl | | F2 | | | 62 | |
| | 15 | B3 | Ali-j1 | | F3 | | | 63 | |
| | 16 | B4 | Ali-j1 | | F4 | Ali-j1 | | 64 | |
| | 17 | B5 | | | F5 | Ali-j1 [Ref] | | 65 | |
| | 18 | B6 | | | F6 | | | 66 | |
| | 19 | B7 | | | F7 | | | 67 | |
| | 20 | B8 | | | F8 | Caf-j1 | | 68 | |
| | 21 | B9 | | | F9 | | | 69 | |
| | 22 | B10 | | | F10 | | | 70 | |
| | 23 | B11 | | | F11 | | | 71 | |
| | 24 | B12 | Caf-j1 (49,44%C) | | F12 | | | 72 | |
| | 25 | C1 | | | G1 | | | 73 | |
| | 26 | C2 | | | G2 | | | 74 | |
| | 27 | C3 | | | G3 | | | 75 | |
| | 28 | C4 | | | G4 | Ali-j1 | | 76 | |
| | 29 | C5 | | | G5 | bl | | 77 | |
| | 30 | C6 | | | G6 | bl | | 78 | |
| | 31 | C7 | | | G7 | Ali-j1 | | 79 | |
| | 32 | C8 | Ali-j1 | | G8 | Ali-j1 | | 80 | |
| | 33 | C9 | Ali-j1 [Ref] | | G9 | | | 81 | |
| | 34 | C10 | | | G10 | | | 82 | |
| | 35 | C11 | | | G11 | | | 83 | |
| | 36 | C12 | Caf-j1 | | G12 | | | 84 | |
| | 37 | D1 | | | H1 | | | 85 | |
| | 38 | D2 | | | H2 | | | 86 | |
| | 39 | D3 | | | H3 | | | 87 | |
| | 40 | D4 | | | H4 | | | 88 | |
| | 41 | D5 | | | H5 | | | 89 | |
| | 42 | D6 | | | H6 | | | 90 | |
| | 43 | D7 | | | H7 | | | 91 | |
| | 44 | D8 | Ali-j1 | | H8 | | | 92 | |
| | 45 | D9 | bl | | H9 | | | 93 | |
| | 46 | D10 | bl | | H10 | | | 94 | |
| | 47 | D11 | Ali-j1 | | H11 | | | 95 | |
| | 48 | D12 | Ali-j1 | | H12 | Ali-j1 | | 96 | |

Figure 4. Typical loading list for sequential isotopic analysis of samples and reference materials in a prescribed fashion. The 96 positions ('line') on this list represent samples for three carousels with 32 positions each.

| sample # | spec.-no. | weight [mg] | area [Vs] | $\delta^{13}\text{C}$ vs.VPDB | area blk [Vs] | $\delta^{13}\text{C}$ -blk vs.PDB | $\delta^{13}\text{C}$ blk-corr | *for corr | avgStd | $\delta^{13}\text{C}$ ref. | Diff-Std | $\delta^{13}\text{C}$ vs.VPDB corr. | avgStd $\delta^{13}\text{C}$ | std.dev. |
|----------|-----------|----------------|--------------|----------------------------------|------------------|--------------------------------------|-----------------------------------|-----------|--------|-------------------------------|----------|---|---------------------------------|----------|
| Ali-J1 | 794 | 0.15 | 198.36 | -34.03 | 1.00 | -26.84 | -34.07 | | | | | -33.99 | | |
| Ali-J1 | 795 | 0.136 | 152.80 | -34.07 | 1.00 | -26.84 | -34.12 | | | | | -34.05 | | |
| Ali-J1 | 796 | 0.117 | 142.76 | -34.11 | 1.00 | -26.84 | -34.16 | * | | | | -34.09 | | |
| 36-246 | 797 | 1.775 | 122.17 | -24.50 | 1.00 | -26.84 | -24.48 | | | | | -24.41 | | |
| 36-246 | 798 | 1.741 | 116.34 | -24.46 | 1.00 | -26.84 | -24.44 | | | | | -24.37 | | |
| Caf-J1 | 799 | 0.229 | 189.53 | -52.05 | 1.00 | -26.84 | -52.19 | | | | | -52.12 | | |
| 36-246 | 800 | 1.731 | 114.68 | -24.48 | 1.00 | -26.84 | -24.45 | | | | | -24.38 | | |
| 16-98-2 | 801 | 1.786 | 86.72 | -22.85 | 1.00 | -26.84 | -22.80 | | | | | -22.73 | | |
| 16-98-2 | 802 | 1.804 | 86.26 | -22.79 | 1.00 | -26.84 | -22.75 | | | | | -22.68 | | |
| 16-98-2 | 803 | 1.795 | 83.75 | -22.84 | 1.00 | -26.84 | -22.79 | | | | | -22.72 | | |
| 114-806 | 804 | 1.776 | 170.37 | -28.00 | 1.00 | -26.84 | -28.00 | | | | | -27.93 | | |
| 114-806 | 805 | 1.852 | 176.49 | -28.08 | 1.00 | -26.84 | -28.09 | | | | | -28.02 | | |
| 114-806 | 806 | 1.795 | 176.36 | -28.07 | 1.00 | -26.84 | -28.08 | | | | | -28.00 | | |
| Ali-J1 | 807 | 0.146 | 170.36 | -33.99 | 1.00 | -26.84 | -34.04 | * | | | | -33.97 | | |
| blk | 808 | 0 | 1.01 | -27.48 | | | | | | | | | | |
| blk | 809 | 0 | 0.98 | -26.20 | | | | | | | | | | |
| Ali-J1 | 810 | 0.144 | 165.17 | -33.98 | 1.00 | -26.84 | -34.02 | * | | | | -33.95 | | |
| Ali-J1 | 811 | 0.133 | 156.46 | -33.89 | 1.00 | -26.84 | -33.93 | * | | | | -33.86 | | |
| 129-504 | 812 | 1.837 | 157.67 | -31.10 | 1.00 | -26.84 | -31.13 | | | | | -31.06 | | |
| 129-504 | 813 | 1.912 | 164.89 | -31.09 | 1.00 | -26.84 | -31.12 | | | | | -31.05 | | |
| 129-504 | 814 | 1.843 | 157.83 | -31.15 | 1.00 | -26.84 | -31.18 | | | | | -31.11 | | |
| 52-368 | 815 | 1.765 | 142.51 | -27.28 | 1.00 | -26.84 | -27.28 | | | | | -27.21 | | |
| 52-368 | 816 | 1.837 | 153.82 | -27.38 | 1.00 | -26.84 | -27.38 | | | | | -27.31 | | |
| 52-368 | 817 | 1.813 | 149.45 | -27.29 | 1.00 | -26.84 | -27.29 | | | | | -27.22 | | |
| 93-668 | 818 | 1.813 | 288.98 | -31.20 | 1.00 | -26.84 | -31.21 | | | | | -31.14 | | |
| 93-668 | 819 | 1.814 | 280.80 | -31.10 | 1.00 | -26.84 | -31.11 | | | | | -31.04 | | |
| 93-668 | 820 | 1.784 | 290.18 | -30.91 | 1.00 | -26.84 | -30.92 | | | | | -30.85 | | |
| 138-390 | 821 | 1.819 | 169.71 | -27.40 | 1.00 | -26.84 | -27.41 | | | | | -27.34 | | |
| 138-390 | 822 | 1.931 | 179.12 | -27.44 | 1.00 | -26.84 | -27.45 | | | | | -27.38 | | |
| 138-390 | 823 | 1.801 | 167.01 | -27.39 | 1.00 | -26.84 | -27.39 | | | | | -27.32 | | |
| 100-727 | 824 | 1.946 | 265.80 | -29.64 | 1.00 | -26.84 | -29.65 | | | | | -29.58 | | |
| 100-727 | 825 | 1.871 | 256.98 | -29.17 | 1.00 | -26.84 | -29.18 | | | | | -29.11 | | |
| 100-727 | 826 | 1.956 | 255.76 | -29.35 | 1.00 | -26.84 | -29.36 | | | | | -29.29 | | |
| Ali-J1 | 827 | 0.14 | 156.33 | -33.86 | 1.00 | -26.84 | -33.90 | * | -34.01 | -33.94 | 0.07 | -33.83 | -33.94 | 0.10 |

Figure 5. Spreadsheet calculation of data from the irm-EAMS system showing the evaluation of the raw data to the final δ -values on an international scale. See text for further explanations.

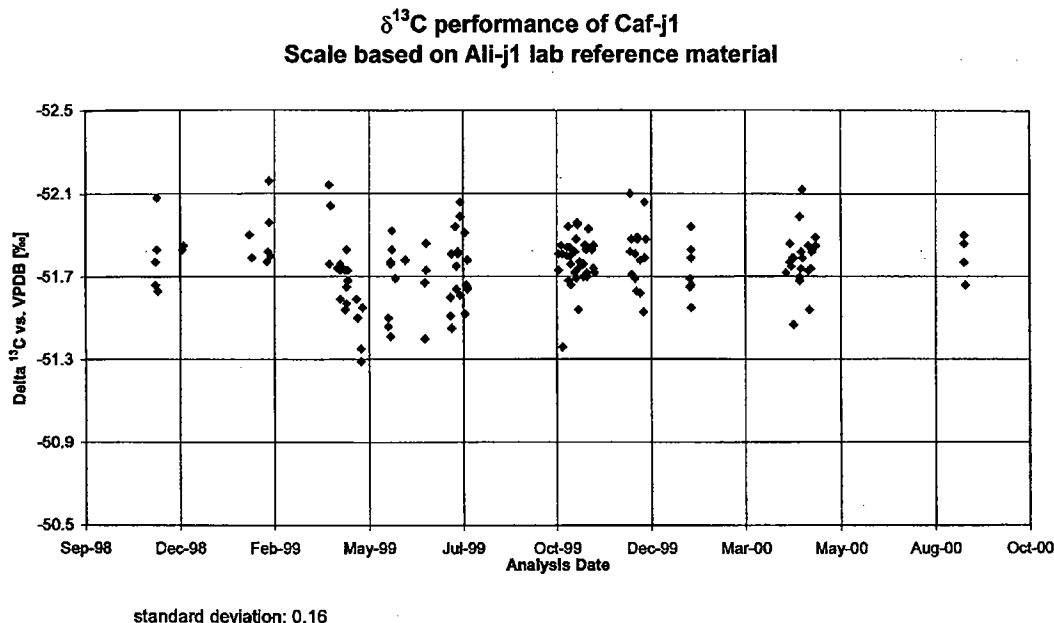


Figure 6. Performance Chart. $\delta^{13}\text{C}$ -values of a quality control standard as a function of measurement time. The scale is based on reference material that went through the same preparation channel as the QA standard. Each sequence of 32 samples measured holds one QA standard that is represented by a single point.

standardized spreadsheet (Fig. 5) for assigning the final δ -values on the respective international scale.

The spreadsheet evaluation³⁷ starts with the original values directly transferred from the host computer ('ISO-DAT' from Finnigan MAT). Columns 1 to 5 (Fig. 5) hold information about the sample including the sample name and weight, the integrated peak area and the measured $\delta^{13}\text{C}$ -value based on the injected reference gas. The average peak area (Vsec) and δ -value (‰) of the blank measurement[†] are used to correct the data for blank contribution applying a simple mass balance correction.

$$\delta^{13}\text{C}_{\text{tot}} \times \text{area}_{\text{tot}} = \delta^{13}\text{C}_{\text{sa}} \times \text{area}_{\text{sa}} + \delta^{13}\text{C}_{\text{blk}} \times \text{area}_{\text{blk}} \quad (3)$$

The suffixes in Eqn. (3) refer to the total (tot), sample (sa) and blank (blk) properties, respectively.

Usually the correction is very small. The data in column 8 (Fig. 5) represent the blank-corrected preliminary $\delta^{13}\text{C}$ -values (‰) when the reference gas is used for standardization. The following columns denote the laboratory standards selected for further correction and final positioning of the data onto the VPDB scale. Ali-j1 (Acetanilide-Jena1) has been determined directly versus NBS-22 oil and USGS24 graphite a number of times and has been assigned a $\delta^{13}\text{C}$ -value of -33.94‰ on the VPDB scale. The measured average $\delta^{13}\text{C}$ -value of the selected Ali-j1 samples in Fig. 5 is -34.01‰ . Thus, there is an offset of 0.07‰ to the accepted δ -value that

[†] We found that the carbon blank in our laboratory mostly has a $\delta^{13}\text{C}$ -value around -25‰ vs. VPDB. The major contributions are carbon in the tin containers and memory in the combustion reactor. Often the blank area is so small that the $\delta^{13}\text{C}$ measurement is erroneous (outside the natural abundance range). In such cases we replace the wrong $\delta^{13}\text{C}$ -value of the blank by our average value, -25‰ .

is used to correct all data (Eqn. (2)). The data in column 13 are the final $\delta^{13}\text{C}$ -values on the VPDB scale. The last two columns are average and precision of the selected Ali-j1 data.

In principle, this procedure represents a one-point calibration of the $\delta^{13}\text{C}$ -values; our scale expansion factor is 1.000. For measuring larger differences in isotopic composition it will be necessary to include a scaling factor in the calculations (see Fig. 10 and the discussion in 'Further corrections').

Not shown in Fig. 5 are further routine calculations of the carbon elemental contents based on the measured peak area and the known carbon content of the reference material (Ali-j1, 71.09% C). The $\delta^{15}\text{N}$ analyses are made in a completely analogous fashion.

Generation of performance charts

We use one dedicated sample position for determining a QA standard (quality assurance), in this case Caf-j1 (a caffeine sample from a 'Traube synthesis' in larger supply), in every sample carousel we run. We have chosen this material because it is off the usual $\delta^{13}\text{C}$ values for C_3 -plants by about -20‰ . With this QA standard, which does not enter the reference calculation, the long-term performance of the irm-EAMS line has been monitored (Fig. 6 for $\delta^{13}\text{C}$ and Fig. 7 for $\delta^{15}\text{N}$).

Figures 6 and 7 are representative for all measurements made on our irm-EAMS systems (in total from three different mass spectrometers and two different interfaces) since we started routine analyses. We measured both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from the same sample until April 1999. From the performance charts we noticed that the accuracy, especially for $\delta^{15}\text{N}$, was not satisfactory and needed improvement. Careful analysis of the data indicated that the problems were

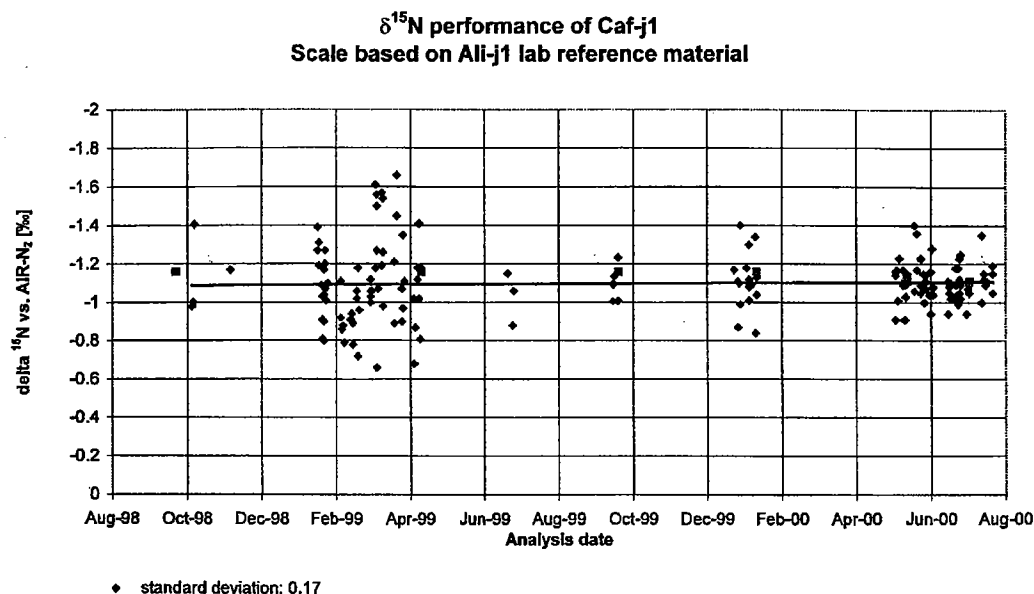


Figure 7. Performance Chart: Same as Fig. 6 for $\delta^{15}\text{N}$ -values

caused by tailing of the CO_2 peak into the nitrogen chromatogram of the subsequent sample. Most materials analyzed had an elemental content of $\leq 2\%$ nitrogen and $\geq 40\%$ carbon. For analysis the CO_2 had to enter the GC column (Porapak Q) and, due to the relatively large amount, was still present and declining steadily during the subsequent nitrogen analysis. CO_2^{++} ions undergo unimolecular decay in the ion source resulting in a $10\% \text{CO}^+$ contribution at m/z 28 and 29 that interferes with N_2^{++} and has a very different isotopic signature. The background assignments of all peaks in the nitrogen chromatogram were affected and hence precision and accuracy suffered. As a consequence, we decided to stop simultaneous isotope ratio measurement

using irm-EAMS altogether. For $\delta^{15}\text{N}$ determination we now trap CO_2 in a chemical (Ascarite) trap installed between the water trap and the GC column of the elemental analyzer.

The performance or quality chart is also the perfect tool to monitor the status of the laboratory reference materials. Any contamination in either the laboratory standard or the QA standard will show up as a step. Slow long-term alteration (e.g. evaporative loss in water standards) will show as a drift. We recommend using such charts as a general tool for maintaining and proving analytical performance in every isotope laboratory for every type of analysis.

In order to demonstrate the effect of the rigorous implementation of the IT principle, we have compiled the

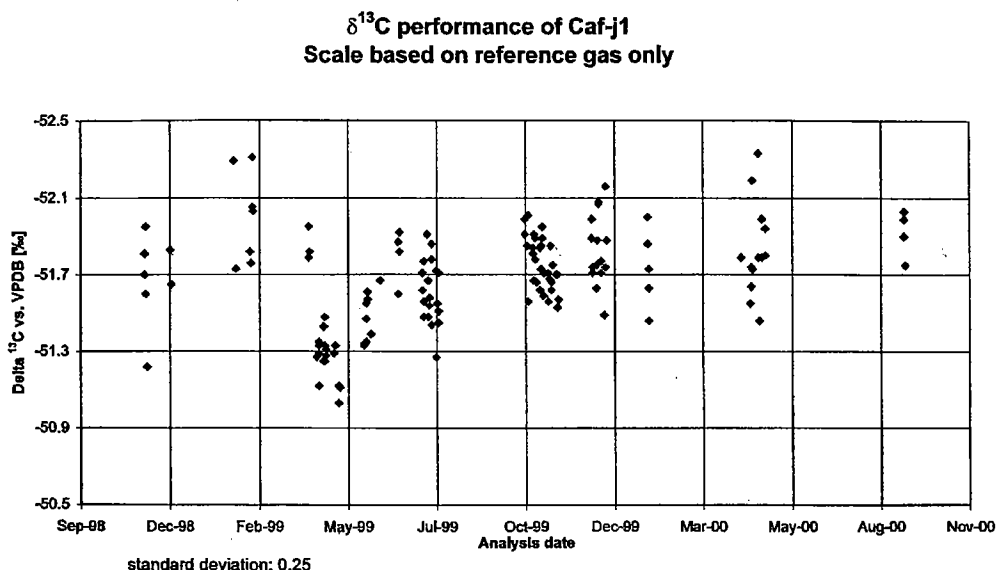


Figure 8. Performance Chart. $\delta^{13}\text{C}$ -values as in Fig. 6. Here, the scale is based on reference gas injections only. Please note the enhanced scatter of the data.

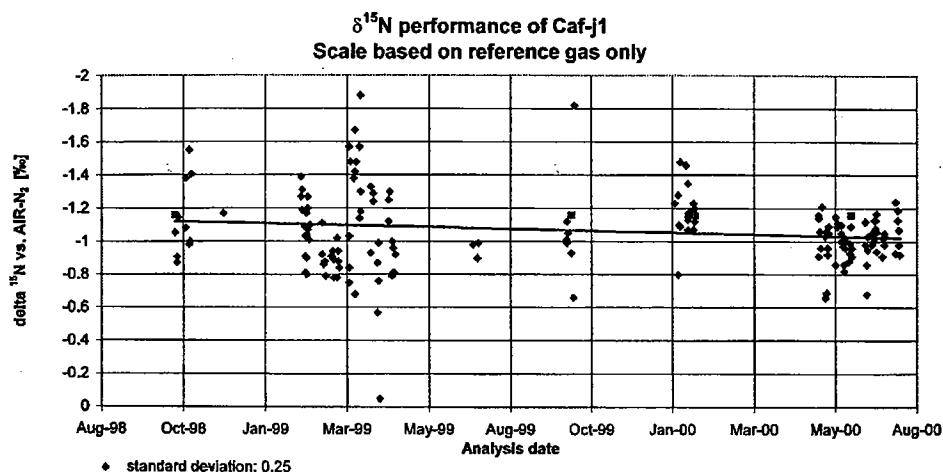


Figure 9. Performance Chart. $\delta^{15}\text{N}$ -values as in Fig. 7. The scale is solely based on reference gas. Compared with Fig. 7 a larger scatter and some outliers are evident.

results of our QA standard based exclusively on our reference gas (Figs. 8 and 9). The data clearly exhibit a larger scatter than the data compiled including the IT calculations. For both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, the precision has increased by about 50%. It is also worth noting that a number of marked outliers are present in Fig. 9 that are not seen in Fig. 7. These outliers might have escaped our attention if the IT principle had not been included in the measurement and evaluation.

The composition of the N_2 gas inside our 50-L high-pressure cylinders has not changed much over the reported time span. There is a slope of 0.04‰/a in the solid line in Fig. 9 that is not present in Fig. 7. This slope probably represents a change in the N_2 reference gas. To explain the differences between the graphs we suspect a number of sources of error that all contribute to the observed dependence of the data:

- the pressure regulator on the tank is critical. Most regulators alter the isotopic composition of the dispensed gas at least temporarily, the size of the alteration being dependent on the gas itself and on the surfaces inside the regulator.³⁸ The same applies to the second type of GC regulators (Porter) in the interface. The alteration for the isotopic composition of CO_2 is in the sub-per mill range.
- The combustion and (or) the reduction tube can be contaminated with previous sample material giving rise to memory effects.
- The combustion efficiency may vary slightly thereby altering the isotopic composition.
- The efficiency of the reduction tube for scavenging excess oxygen and for reducing nitrous oxides may change with time.
- When diluting the CO_2 effluent, a change in isotopic composition may occur due to diffusion effects.³⁵

As long as the reference material suffers from the same deficiencies during preparation, the errors will at least tend to cancel out and the long-term data monitored by a QA standard will exhibit much better laboratory performance than with pure standard gas referencing.

D/H determination from water

Measurement of δD -values from water samples is carried out on a fully automated chromium reduction system at 900°C ('H/Device', Finnigan MAT, Bremen, Germany) directly coupled to the sample inlet of the dual inlet system of our MAT 252 isotope ratio MS.³⁹ The samples, positioned in a GC autosampler tray (AS200, CTC Analytics, Switzerland), are transferred to the hot injection port via a gas-tight syringe (Model 702, Hamilton, Switzerland). The quartz reactor (Finnigan MAT) is filled with chromium powder (100 mesh, Alfa Aesar, Karlsruhe, Germany). The reaction of water vapor with the hot chromium is almost instantaneous. The valve at the end of the reactor is opened after a preset time and the H_2 gas is transferred to an intermediate equilibration volume. Again, after a preset equilibration time (in order to avoid diffusive alteration of the isotopic composition of the hydrogen gas), the reactor valve is closed and the H_2 gas is passed to the sample volume of the dual inlet for measurement. The equilibration and transfer times are critical and must be under computer control.

We routinely run 60 analyses (~20 h) in a sequence with the laboratory reference water and the QA standard distributed in a prescribed loading list similar to that presented in Fig. 4. All data enter a spreadsheet for final calculation of δD -values (Fig. 10).

The data in column 3 (Fig. 10) are the original results as automatically transferred from the mass spectrometer PC. All samples are run in triplicate. The data treatment is more elaborate owing to the fact that multiple effects must be accounted for. First, we apply a memory correction (column 4) to the δD -values against the working gas. The magnitude of the correction is 1% carry over from the previous sample plus 0.2% from the sample before. The effect of the memory correction can be judged from the triplets where the difference to the precursor sample is large.

Next is the IT calculation, that is, replacement of the hydrogen gas reference by the laboratory standard water

Siberian Water Samples Spec# 7379-7438, H/Device, Feb. 22, 1999

| # | Name | raw & vs Helm(-220) | memory corrected (1+0.2%) | average | δ ref. gas offset | δ VSMOW | average | 1st drift correction | average | 2nd drift correction | average | base adjusted | average | δ VSMOW / SLAP (+0.7%) | average | stdDev |
|----|----------------------|------------------------|---------------------------------|---------|--------------------------------|-------------------|---------|-------------------------|---------|-------------------------|---------|------------------|---------|-------------------------------------|---------|--------|
| 1 | WWJ-1A/Isolab/ | -63.834 | -63.83 | | | -66.03 | | -66.41 | | -66.41 | | -66.41 | | -66.41 | | |
| 2 | WWJ-1A/Isolab/ | -63.932 | -63.93 | | | -66.13 | | -66.38 | | -66.38 | | -66.38 | | -66.38 | | |
| 3 | WWJ-1A/Isolab/ | -64.3018 | -64.31 | | | -66.50 | | -66.63 | | -66.63 | | -66.63 | | -66.63 | | |
| 4 | WWJ-1A/Isolab/ | -64.3116 | -64.31 | | | -66.51 | | -66.51 | | -66.51 | | -66.51 | | -66.51 | | |
| 5 | WWJ-1A/Isolab/ | -64.3122 | -64.31 | | | -66.51 | | -66.39 | | -66.39 | | -66.39 | | -66.39 | | |
| 6 | WWJ-1A/Isolab/ | -64.3971 | -64.40 | -64.25 | -2.35 | -66.60 | -66.45 | -66.35 | -66.45 | -66.35 | -66.45 | -66.35 | -66.45 | -66.35 | -66.45 | 0.11 |
| 7 | 142 A/E.D.Schulze/ | -115.955 | -116.57 | | | -118.65 | | -118.28 | | -118.28 | | -118.28 | | -118.28 | | |
| 8 | 142 A/E.D.Schulze/ | -117.533 | -117.66 | | | -119.73 | | -119.23 | | -119.23 | | -119.23 | | -119.23 | | |
| 9 | 142 A/E.D.Schulze/ | -116.798 | -116.79 | | | -118.87 | -119.08 | -118.24 | -118.58 | -118.24 | -118.58 | -118.24 | -118.58 | -118.61 | -118.95 | 0.56 |
| 10 | 150/E.D.Schulze/ | -133.435 | -133.63 | | | -135.67 | | -134.92 | | -134.92 | | -134.92 | | -134.92 | | |
| 11 | 150/E.D.Schulze/ | -134.854 | -134.90 | | | -136.94 | | -136.07 | | -136.07 | | -136.07 | | -136.07 | | |
| 12 | 150/E.D.Schulze/ | -134.09 | -134.08 | | | -136.12 | -136.24 | -135.12 | -135.37 | -135.12 | -135.37 | -135.12 | -135.37 | -135.60 | -135.85 | 0.61 |
| 13 | 131/E.D.Schulze/ | -91.6047 | -91.09 | | | -93.23 | | -92.11 | | -92.11 | | -92.11 | | -92.11 | | |
| 14 | 131/E.D.Schulze/ | -91.9463 | -91.87 | | | -94.00 | | -92.75 | | -92.75 | | -92.75 | | -92.75 | | |
| 15 | 131/E.D.Schulze/ | -91.8027 | -91.80 | | | -93.93 | -93.72 | -92.57 | -92.48 | -92.57 | -92.48 | -92.57 | -92.48 | -92.75 | -92.68 | 0.33 |
| 16 | 106 A/E.D.Schulze/ | -129.781 | -130.24 | | | -132.28 | | -130.79 | | -130.79 | | -130.79 | | -130.79 | | |
| 17 | 106 A/E.D.Schulze/ | -130.974 | -131.06 | | | -133.10 | | -131.49 | | -131.49 | | -131.49 | | -131.49 | | |
| 18 | 106 A/E.D.Schulze/ | -131.055 | -131.06 | | | -133.10 | -132.83 | -131.36 | -131.21 | -131.36 | -131.21 | -131.36 | -131.21 | -131.81 | -131.68 | 0.38 |
| 19 | RWB-J1/Isolab/ | -7.87838 | -6.40 | | | -8.73 | | -8.87 | | -8.87 | | -8.87 | | -8.87 | | |
| 20 | RWB-J1/Isolab/ | -6.72937 | -6.47 | | | -8.80 | | -8.81 | | -8.81 | | -8.81 | | -8.81 | | |
| 21 | RWB-J1/Isolab/ | -6.59651 | -6.59 | | | -8.93 | -8.82 | -8.81 | -6.83 | -8.81 | -6.83 | -8.81 | -6.83 | -8.81 | -6.83 | 0.03 |
| 22 | 134 A/E.D.Schulze/ | -119.087 | -120.44 | | | -122.50 | | -120.26 | | -120.26 | | -120.26 | | -120.26 | | |
| 23 | 134 A/E.D.Schulze/ | -120.452 | -120.69 | | | -122.76 | | -120.40 | | -120.40 | | -120.40 | | -120.40 | | |
| 24 | 134 A/E.D.Schulze/ | -121.628 | -121.64 | | | -123.71 | -122.99 | -121.22 | -120.63 | -121.22 | -120.63 | -121.22 | -120.63 | -121.60 | -121.01 | 0.52 |
| 25 | 151 A/E.D.Schulze/ | -120.951 | -120.95 | | | -123.01 | | -120.40 | | -120.40 | | -120.40 | | -120.40 | | |
| 26 | 151 A/E.D.Schulze/ | -121.147 | -121.15 | | | -123.21 | | -120.48 | | -120.48 | | -120.48 | | -120.48 | | |
| 27 | 151 A/E.D.Schulze/ | -121.266 | -121.27 | | | -123.33 | -123.18 | -120.47 | -120.45 | -120.47 | -120.45 | -120.47 | -120.45 | -120.85 | -120.83 | 0.04 |
| 28 | 115/E.D.Schulze/ | -92.6989 | -92.36 | | | -94.49 | | -91.50 | | -91.50 | | -91.50 | | -91.50 | | |
| 29 | 115/E.D.Schulze/ | -92.8674 | -92.81 | | | -94.94 | | -91.83 | | -91.83 | | -91.83 | | -91.83 | | |
| 30 | 115/E.D.Schulze/ | -92.8432 | -92.84 | | | -94.97 | -94.80 | -91.74 | -91.69 | -91.74 | -91.69 | -91.74 | -91.69 | -91.92 | -91.87 | 0.17 |
| 31 | 144 A/E.D.Schulze/ | -144.125 | -144.74 | | | -146.75 | | -143.39 | | -143.39 | | -143.39 | | -143.39 | | |
| 32 | 144 A/E.D.Schulze/ | -144.58 | -144.69 | | | -146.70 | | -143.21 | | -143.21 | | -143.21 | | -143.21 | | |
| 33 | 144 A/E.D.Schulze/ | -144.492 | -144.49 | | | -146.50 | -146.65 | -142.89 | -143.17 | -142.89 | -143.17 | -142.89 | -143.17 | -143.43 | -143.70 | 0.25 |
| 34 | WWJ-1A/Isolab/ | -68.8824 | -67.97 | | | -70.16 | | -66.43 | | -66.43 | | -66.43 | | -66.43 | | |
| 35 | WWJ-1A/Isolab/ | -68.45 | -68.29 | | | -70.48 | | -66.63 | | -66.63 | | -66.63 | | -66.63 | | |
| 36 | WWJ-1A/Isolab/ | -68.0867 | -68.08 | | | -70.27 | -70.31 | -66.29 | -66.45 | -66.30 | -66.45 | -66.30 | -66.45 | -66.29 | -66.45 | 0.17 |
| 37 | 145/E.D.Schulze/ | -139.372 | -140.23 | | | -142.25 | | -138.14 | | -138.15 | | -138.15 | | -138.15 | | |
| 38 | 145/E.D.Schulze/ | -140.829 | -140.99 | | | -143.01 | | -138.78 | | -138.79 | | -138.79 | | -138.79 | | |
| 39 | 145/E.D.Schulze/ | -140.944 | -140.95 | | | -142.97 | -142.74 | -138.61 | -138.51 | -138.63 | -138.52 | -138.63 | -138.52 | -139.14 | -139.03 | 0.34 |
| 40 | 1 NB 23/E.D.Schulze/ | -72.4351 | -71.61 | | | -73.79 | | -69.32 | | -69.34 | | -69.34 | | -69.34 | | |
| 41 | 1 NB 23/E.D.Schulze/ | -71.4826 | -71.33 | | | -73.52 | | -68.91 | | -68.94 | | -68.94 | | -68.94 | | |
| 42 | 1 NB 23/E.D.Schulze/ | -71.5855 | -71.58 | | | -73.77 | -73.69 | -69.04 | -69.09 | -69.07 | -69.12 | -69.07 | -69.12 | -69.09 | -69.14 | 0.20 |
| 43 | 144/E.D.Schulze/ | -146.175 | -147.07 | | | -149.07 | | -144.22 | | -144.26 | | -144.26 | | -144.26 | | |
| 44 | 144/E.D.Schulze/ | -147.004 | -147.16 | | | -149.17 | | -144.19 | | -144.23 | | -144.23 | | -144.23 | | |
| 45 | 144/E.D.Schulze/ | -147.476 | -147.48 | | | -149.49 | -149.24 | -144.39 | -144.27 | -144.43 | -144.31 | -144.43 | -144.31 | -144.98 | -144.85 | 0.11 |
| 46 | 1 NB 19/E.D.Schulze/ | -59.8333 | -58.78 | | | -60.99 | | -55.77 | | -55.82 | | -55.82 | | -55.82 | | |
| 47 | 1 NB 19/E.D.Schulze/ | -58.7421 | -58.55 | | | -60.76 | | -55.42 | | -55.47 | | -55.47 | | -55.47 | | |
| 48 | 1 NB 19/E.D.Schulze/ | -58.7176 | -58.72 | | | -60.93 | -60.89 | -55.45 | -55.55 | -55.51 | -55.60 | -55.51 | -55.60 | -55.44 | -55.53 | 0.19 |
| 49 | 145 A/E.D.Schulze/ | -140.411 | -141.39 | | | -143.41 | | -137.81 | | -137.88 | | -137.88 | | -137.88 | | |
| 50 | 145 A/E.D.Schulze/ | -141.207 | -141.38 | | | -143.40 | | -137.68 | | -137.75 | | -137.75 | | -137.75 | | |
| 51 | 145 A/E.D.Schulze/ | -141.676 | -141.68 | | | -143.70 | -143.50 | -137.85 | -137.78 | -137.93 | -137.85 | -137.93 | -137.85 | -138.43 | -138.35 | 0.09 |
| 52 | 150 A/E.D.Schulze/ | -137.474 | -137.42 | | | -139.45 | | -133.48 | | -133.56 | | -133.56 | | -133.56 | | |
| 53 | 150 A/E.D.Schulze/ | -137.517 | -137.51 | | | -139.53 | | -133.44 | | -133.52 | | -133.52 | | -133.52 | | |
| 54 | 150 A/E.D.Schulze/ | -137.563 | -137.56 | | | -139.59 | -139.53 | -133.37 | -133.43 | -133.46 | -133.51 | -133.46 | -133.51 | -133.93 | -133.98 | 0.05 |
| 55 | 112 A/E.D.Schulze/ | -111.57 | -111.26 | | | -113.35 | | -107.00 | | -107.10 | | -107.10 | | -107.10 | | |
| 56 | 112 A/E.D.Schulze/ | -110.883 | -110.82 | | | -112.91 | | -106.44 | | -106.54 | | -106.54 | | -106.54 | | |
| 57 | 112 A/E.D.Schulze/ | -110.981 | -110.98 | | | -113.07 | -113.11 | -106.48 | -106.64 | -106.58 | -106.74 | -106.58 | -106.74 | -106.86 | -107.02 | 0.31 |
| 58 | WWJ-1A/Isolab/ | -71.5395 | -71.07 | | | -73.25 | | -66.53 | | -66.64 | | -66.64 | | -66.64 | | |
| 59 | WWJ-1A/Isolab/ | -71.0069 | -70.92 | | | -73.10 | | -66.26 | | -66.38 | | -66.38 | | -66.37 | | |
| 60 | WWJ-1A/Isolab/ | -71.003 | -71.00 | | | -73.18 | -73.18 | -66.22 | -66.34 | -66.34 | -66.45 | -66.34 | -66.45 | -66.33 | -66.45 | 0.17 |

Figure 10. Spreadsheet calculation of data from the H/Device sample preparation line showing the evaluation of the raw data to the final δ -values on an international scale. See text for further explanations.

samples in the run. The δ D-value (-66.45‰) of WWJ-1A (working water-Jena1A) has been established by direct measurement against VSMOW and SLAP samples (freshly opened ampoules) obtained from the IAEA in Vienna. Using this δ D-value and the actually measured average of WWJ-1A, the apparent working reference gas offset (-2.35‰, Fig.

10) is calculated according to:

$$\text{offset}_{\text{wgas}} = (-66.45 - \text{avg}_{\text{gwwj1}}) / (1 + \text{avg}_{\text{gwwj1}}/1000) \quad (4)$$

This offset is used to correct the raw memory corrected δ D-values and to provide a preliminary positioning relative to VSMOW (Eqn. (2)).

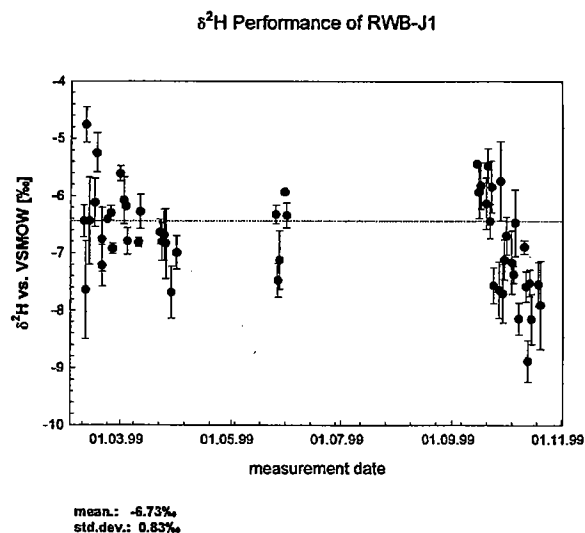


Figure 11. Performance Chart. $\delta^2\text{H}$ -values of the quality control standard RWB-J1 as a function of measurement time.

The next columns constitute a two-pass drift correction. The drift correction is necessary because the hydrogen reference gas in the dual inlet changes isotopic composition over time (a complete 60-sample sequence takes about 20 h). This is due to the fact that length and crimping of the capillaries are a compromise between CO_2 and H_2 work. To avoid back diffusion of isotopically altered gas into the variable volume, the capillaries would have to be twice as long for H_2 . Alternatively, a different crimp on reference vs. sample side could be used so that the reference could be operated at a much higher pressure. The magnitude of the drift depends on the filling state of the reference bellow volume at the beginning of the sequence. We have observed drifts of 3–5‰ in 20 h when starting with a full reservoir. Less reference gas results in larger drifts. Because the drift should follow an exponential function, we have implemented our analysis sequence with three reference water measurements in positions 34 to 36 and three more at the end of the sequence. The results for these reference water injections are used to correct the reference gas drift. A fine adjustment is made in column 13 (Fig. 10) that is only necessary for making the data mathematically consistent after drift correction (in the example shown the data are identical). The last correction involves scaling according to the observed scale contraction.⁴⁰ On average we have measured -425‰ instead of -428‰ for SLAP vs. VSMOW, hence all data are corrected by multiplying the difference from WWJ-1A by 1.007. The last two columns are the final δD -values on the VSMOW/SLAP scale and their precision.

^k Usually pure CO_2 gas is used for equilibration and a mass balance correction according to:

$$\delta_{\text{true}} = \delta_{\text{meas}} + \alpha x_{\text{gas}}(\delta_{\text{meas}} - \delta_{\text{gas}})/x_w \quad (5)$$

is applied (α = exchange fractionation factor, δ_{gas} is the $\delta^{18}\text{O}$ -value of the added CO_2 gas before equilibration and x_{gas} and x_w are the mole fractions of gas and liquid, respectively).

In our case, due to the small amount of CO_2 in the gas phase, the correction can be neglected down to about 50 μL H_2O . Equation (5) also applies to the equilibration of hydrogen gas with water.

In every 60-sample sequence we measure RWB-j1 (reference water B-Jena1) as a QA standard for generating the performance chart associated with the D/H procedure. Figure 11 shows the data as a function of measurement time. The overall precision of 0.83‰ vs. VSMOW is certainly acceptable. There is a tendency to more negative values at the end of the sequence that deserves further attention. During the short period of measurements this is not likely to be caused by a relative drift of our laboratory working waters. However, most of the internal precisions measured are better than 0.8‰ (see also Fig. 10). We attribute this finding to the fact that we do not determine the SLAP/VSMOW scaling factor for every sequence in contrast to frequent measurement of the H_3^+ factor. We started by using water samples with about 60‰ difference for implementing this correction. This difference turned out to be too small in relation to the measurement precision, leading to erroneous scaling factors. On the other hand, measuring water with about -400‰ δD in the same sequence affects the memory correction and the three samples following this negative water would have to be discarded. The potential for improving the performance chart precision to about 0.5‰ is within reach using the technique described.

Equilibration/irm-analysis for $\delta^{18}\text{O}$ determination

The ^{18}O abundance in water samples is usually measured with a modified $\text{CO}_2/\text{H}_2\text{O}$ equilibration technique. With a syringe we inject 400 μL water aliquots into 10-mL glass containers ('Exetainer') topped with a septum. Before water injection the glass vials are filled with a mixture of 0.5% CO_2 in helium.^k Equilibration takes place inside a 96-position autosampler rack held at 30°C ($\pm 0.1^\circ\text{C}$) that is part of the irm interface (GasBench II, Finnigan MAT). We operate the autosampler rack in two separate stages holding 48 sample tubes each. While one sequence of 48 samples is measured, the other batch of 48 samples is allowed to equilibrate for roughly 20 h. With this loading scheme we manage to have the system analyze samples almost continuously, i.e. 6 days per week.

Following equilibration, the sample vial is analyzed by piercing the septum with a double wall needle. The needle has a feed (He) and an exit (sample CO_2 in He). The flushing rate is approximately 0.3 mL/min. The sample gas flows over a Nafion[®] dryer and then through an injection loop from which the GC run (Poraplot Q) is started. From a single sample tube we inject about ten times. The ten CO_2 peaks are evaluated isotopically and the results are transferred to a spreadsheet for further data evaluation.

The measurement sequence is assembled as before, i.e. following a rigid structure determined by a dedicated loading list. Hence, the evaluation follows a similar pattern: a mean $\delta^{18}\text{O}$ is calculated for all CO_2 peaks from a single sample. The δ -values are referenced at first to co-injected CO_2 standard gas peaks that serve as a mediator between the samples. WWJ-1A water samples strategically located in the 48-sample sequence again serve to position the preliminary $\delta^{18}\text{O}$ -values onto the VSMOW scale. As in deuterium analysis, a light reference water sample is used to establish

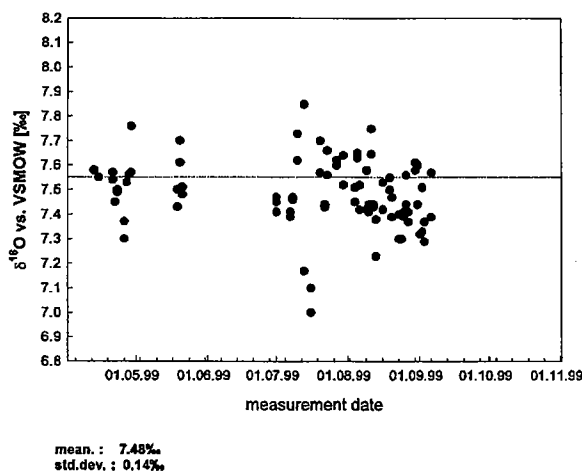
$\delta^{18}\text{O}$ Performance of RWB-J1

Figure 12. Performance Chart. $\delta^{18}\text{O}$ -values of the quality control standard RWB-J1 as a function of measurement time. Analysis is made using an equilibration/irm-GC/MS system (GasBench II, Finnigan MAT).

the final scale normalization so that SLAP returns a value of -55.5‰ vs. VSMOW.

Figure 12 shows the long-term $\delta^{18}\text{O}$ -performance of our RWB-J1 QA reference water. Each point corresponds to a single RWB-J1 sample in a 48-sample sequence. Apart from the scatter of the individual sample measurements, the

¹ If the reference material has been determined ten times in the sequence and the precision of the measurement is 0.1‰ then the isotopic value of the reference is determined with an error of $t \cdot (0.1\text{‰}) / \sqrt{10} = 0.063\text{‰}$. The Student factor t (2.0 in our case) reflects the statistically low number of measurements.

overall precision of 0.14‰ also includes the error of the reference water determination in the respective analysis sequence.¹

Isotopic analysis of CO_2 in air

The isotopic composition of CO_2 in air is important for constraining the sources and sinks of this important greenhouse gas in the atmosphere. The average $\delta^{13}\text{C}$ -value of CO_2 in the atmosphere has remained fairly constant over millions of years at about -6.5‰ vs. VPDB. During the last 200 years anthropogenic input from fossil fuel burning has lead to a decline with an average $\delta^{13}\text{C}$ -value of about -8‰ at present. The decline continues at a rate of about $0.03\text{‰}/\text{a}$. The seasonal cycle of $\delta^{13}\text{C}$ varies with latitude and is a function of photosynthesis as well as human activity. At the South Pole the size of the cycling is about 0.05‰ (peak to peak) and increases to a maximum of 0.8‰ at high northern latitudes.⁴¹ These rather small isotopic signatures comprise multiple components including fossil fuel burning. Their measurement requires a high precision and accuracy analytical setup.

Our extraction line is schematically depicted in Fig. 13. From a Multiport valve the air passes through a capillary with a crimp, over a water trap at -70°C and a trap kept at -196°C . Here, CO_2 is frozen out at a pressure of about 400 mbar. The air is pumped through the pumping lines of the dual inlet system of our MAT 252 mass spectrometer.⁴² Sample CO_2 is measured directly from the sampling reservoir via a crimped capillary to the 'changeover valve'. The system is under full computer control for reliable timing and unattended operation. The precision of the system is aimed at and close to 0.01‰ $\delta^{13}\text{C}$ vs. VPDB and 0.025‰ $\delta^{18}\text{O}$ vs. VSMOW. It is very difficult to routinely prepare CO_2 from the primary reference materials (carbonates) across different laboratories at such levels of accuracy. Hence, the atmospheric CO_2 isotope ratio scales are refined scales that

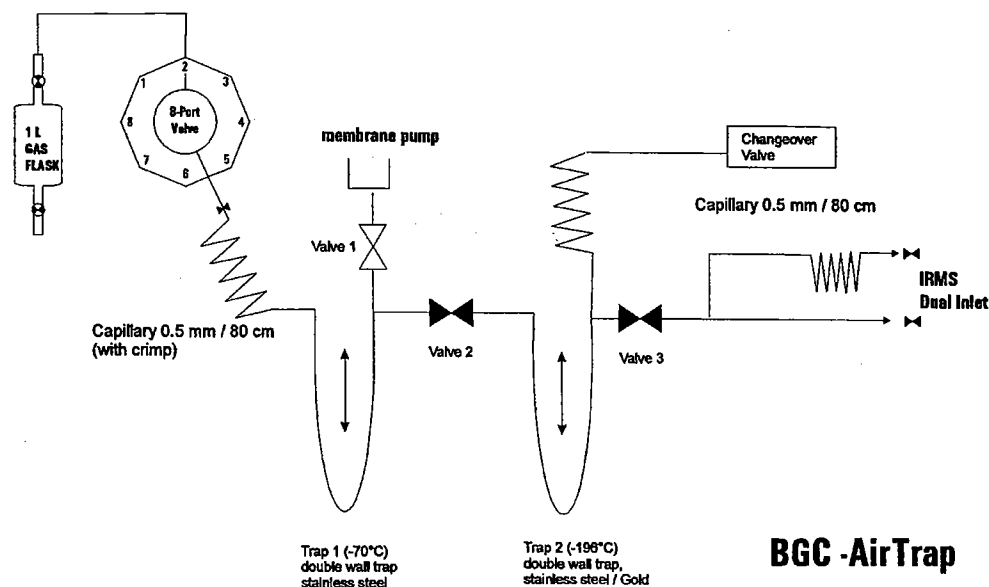


Figure 13. Schematic layout of the automated extraction system ('BGC-Airtrap') for high precision measurement of CO_2 isotope ratios in air.

use air samples and air in high-pressure cylinders as reference material. Consequently, the referencing in our system is made by a combination of permanently attached reference air (Multiport valve positions 1 and 2) as well as air in 1-L flasks that are exchanged routinely with other laboratories. The major problem here derives from the fact that gases are volatile. To keep them as reliable references requires a hierarchy of reference gases and a strategy that automatically detects mutual drifts in order to take corrective action. Without internal referencing high-precision measurement and international comparability of data would not be possible.

O₂/N₂ ratios in air

Complementary to the increase of CO₂ in the atmosphere there is a decrease of O₂. For every carbon atom burnt from fossil fuel one O₂ molecule is lost to CO₂. O₂ has sources and sinks that differ greatly from those of CO₂. Thus, by studying the decrease of O₂ i.e. measuring the O₂/N₂ ratio with high precision, a great deal can be learned about the global carbon cycle including the partitioning of sinks for CO₂ between the oceans and the land biosphere.

We have built a mass spectrometric inlet system for measuring O₂/N₂ ratios with a precision at the 5 perMeg^m level that is capable of monitoring small changes in atmospheric O₂ concentration. The system includes a 16-connection Multiport valve and an open split which is fed alternately from a sample and a reference gas, both switched on and off from a common transfer point, to a Delta⁺XL isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany). Referencing is made in a very similar fashion to that for CO₂ in air by measuring versus an air reference and implementing a multiple referencing hierarchy system. There is no international scale for this kind of work. Currently, consistency in the data is achieved by matching the scales of longer-term records measured by different laboratories. An intensive cooperation to overcome these limits to the comparability of data is under way by a group of laboratories involved in this type of analysis.

Correction of isobaric interferences

A review of referencing in isotope ratio measurements would not be complete without a discussion of the necessary corrections to the raw data. The precise measurement of isotope ratios is often hampered by interfering ion currents from other species hitting the same Faraday cup detectors. Among the most prominent examples are the contribution of

H₃⁺ on the mass 3 channel where δD is measured and the isobaric ¹²C¹⁸O¹⁷O⁺ ion current at the mass 45 position where $\delta^{13}C$ from CO₂ is determined. Here we intend to summarize some of the more important corrections. Further information for in-depth treatment is given in the cited literature.

¹⁷O correction for $\delta^{13}C$ determination

Using CO₂ gas, $\delta^{13}C$ cannot be measured independent of the oxygen isotope ratio unless the mass spectrometer used provides a working mass resolution of >52000. CO₂ gas contains a number of species with different masses ('isotopomers'). All combinations of stable isotopes are present, some of them very low in abundance. The list of isotopomers includes the major species ¹²C¹⁶O¹⁶O (*m/z* 44), ¹³C¹⁶O¹⁶O and ¹²C¹⁷O¹⁶O (*m/z* 45), ¹²C¹⁶O¹⁸O (*m/z* 46), as well as the minor species ¹³C¹⁷O¹⁶O and ¹²C¹⁷O¹⁷O (*m/z* 46), ¹³C¹⁷O¹⁷O, ¹³C¹⁸O¹⁶O and ¹²C¹⁷O¹⁸O (*m/z* 47), ¹²C¹⁸O¹⁸O and ¹³C¹⁷O¹⁸O (*m/z* 48) and ¹³C¹⁸O¹⁸O (*m/z* 49). When working in the natural abundance range of the isotopes the contributions of the minor species is small enough to be neglected. The ¹⁷O moiety at *m/z* 45, however, has a 7% contribution to the mass 45 ion current and must always be corrected for when determining $\delta^{13}C$ -values.

The square-root formula for correcting the ¹⁷O contribution to the 45/44 ratio is sometimes referred to as the Craig correction. Harmon Craig⁹ assumed a tight relationship between ¹⁷O and ¹⁸O with a fractionation coefficient λ of 0.5 exactly and devised a simple correction expression:

$$\delta^{13}C = 1.0676^{45} \delta_{\text{meas}} - 0.0338 \delta^{18}O \quad (6)$$

The numerical values in this expression are the ratio terms ⁴⁵R/¹³R and ¹⁷R/(²¹³R) of the reference gas evolved from the PDB standard as determined or used by Craig. As explicitly pointed out in the paper these numerical values do not apply to other standards and must therefore be used with great care.ⁿ Moreover, the average relation of ¹⁷O and ¹⁸O on earth (Craig equation: (¹⁸R_{sa}/¹⁸R_{st})^λ = ¹⁷R_{sa}/¹⁷R_{st}) cannot be described exactly using a fractionation factor λ of 0.5. Instead, 0.516⁴³ seems to be closer to the correct value for λ .^o The debate about the exact relationship found on earth is ongoing with new experimental values of 0.524 and 0.528 reported for λ .^{44,45}

PDB has been superseded by VPDB and the absolute ratios of the reference scale have been refined. In order to keep literature data consistent, Allison *et al.*⁴⁶ have compiled a recommendation for the ¹⁷O correction that keeps the 0.5 fractionation factor and provides exact values for all ratios involved. On the other hand, Santrock *et al.*⁴⁷ have shown in a H₂O/CO₂ equilibration experiment that data for $\delta^{13}C$ were not independent of the $\delta^{18}O$ -values of the equilibration water when using the Craig equation. The correction proposed includes a fractionation factor of 0.516 and an iterative correction procedure because the exact equations cannot be solved analytically for ¹³C. The dispute is not yet settled. Whatever correction algorithm is chosen (the mass spectrometer software should provide a choice), it is important to make sure that the reference gas isotope ratios

^m 1 perMeg is 0.001‰ in δ -units. It corresponds to a concentration change of 0.2 ppm O₂ in air.

ⁿ The numerical values in Eqn. (6) may be treated as variables. In this case, a set of water equilibration experiments with CO₂ gas that has identical carbon but different oxygen isotope ratios and vice versa can be made to determine the values of these variables. A correction based on these values will result in correct $\delta^{13}C$ -values irrespective of the exact knowledge of the oxygen isotope fractionation factors (T.B. Coplen, private communication).

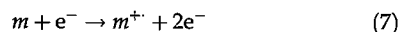
^o If the kinetics of the reactions causing isotopic fractionation are governed entirely by the respective zero point energy differences it can be shown that $\lambda = [(\mu_{16})^{-0.5} - (\mu_{17})^{-0.5}] / [(\mu_{16})^{-0.5} - (\mu_{18})^{-0.5}]$, with μ being the reduced mass. Using exact masses for a C-O bond the result is $\lambda = 0.5273$ which represents an upper limit for kinetic processes. In equilibrium processes, the value for λ can be as high as 0.531.⁴³

used in the calculations are consistent with the $\delta^{13}\text{C}$ -values assigned to the primary reference material.

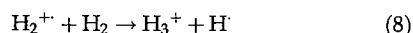
Please note that the correction is only valid for CO_2 from terrestrial sources, not for tracer studies. In addition, there is a strong ^{17}O anomaly in ozone in the stratosphere that affects other trace gases including CO_2 sampled from the atmosphere.

H_3^+ correction

Inside the ion source of the mass spectrometer ions are produced from neutral molecules m by 70 eV electron impact according to:



The molecular radical cation m^{+} is produced in a variety of excited states. If the internal energy is high enough, molecular ions may decompose in a unimolecular decay reaction resulting in a daughter ion and a neutral (mostly a radical) giving rise to the observed mass spectrum. Another process can take place when ions within the ion source hit a neutral molecule. In this case, secondary ions may be formed via ion/molecule reactions. The formation of H_3^+ follows such a mechanism:



The reaction constant of Eqn. (8) is proportional to the number of both H_2^{+} and H_2 . For a given sensitivity of the mass spectrometer, the number of H_2 molecules is proportional to the H_2^{+} ion current, hence:

$$[\text{H}_3^{+}] = k \times [\text{H}_2^{+}]^2 \quad (9)$$

and the ratio $[\text{H}_3^{+}]/[\text{H}_2^{+}]$ is a linear function of the mass 2 ion current ($= k \times [\text{H}_2^{+}]$). The proportionality constant k is called the ' H_3^+ factor'. It is conveniently expressed in (ppm/nA) units.

In practice, the ion source is tuned to a large acceleration field across the ionization volume in order to minimize the time for the ion/molecule reaction (8) and hence suppress H_3^+ production. The H_3^+ factor is measured by observing the mass 3 ion current as a function of mass 2 ion intensity; a linear function is fitted through the data points. The correction involves a simple point-to-point subtraction of a linear portion of the mass 2 ion current from the signal observed at mass position 3. This is usually performed using the (clean) working reference gas. In particular, when helium carrier gas techniques are used, there may be other sources of non-linearity of m/z 3/2 including isobaric interference of He^{2+} ions at m/z 2 that should be studied and evaluated separately or traces of hydrocarbons that constitute a different source for H_3^+ ions.

There are other means of correcting the contribution of H_3^+ to the measured 3/2 ion current ratio.⁴⁰ The simplest correction is no correction at all which will obviously result in differences between reference and sample that are too small. Provided there are several isotopically known samples interspersed with the analyte material, a correction can be deduced from the results of these that may fully cover the requirements. As a general guideline, however, we feel that all effects should be studied and quantified as precisely

as possible in order to apply a valid correction and keep track of the details.

^{18}O correction for $\delta^{34}\text{S}$ determination using SO_2 gas

As for carbon isotope ratio analysis, the ion currents of SO_2 are influenced by the oxygen isotopes. In order to extract $\delta^{34}\text{S}$ the $^{32}\text{S}^{16}\text{O}^{18}\text{O}^{+}$ contribution to the mass 66 ion current (about 8.3%) needs to be taken into account. Unfortunately, there is no independent measurement of the other oxygen isotope, ^{17}O , which would enable a strategy similar to that for carbon isotope ratios. Instead, the established correction aims at keeping the oxygen isotopes in the sample and the reference SO_2 identical. In this case, the measured δ^{66} -values can be converted to $\delta^{34}\text{S}$ -values according to:⁴⁸

$$\delta^{34}\text{S} = {}^{66}\delta_{\text{meas}} + 2^{18}\text{R}/^{34}\text{R} \times ({}^{66}\delta_{\text{meas}} - \Delta\delta^{18}\text{O}) \quad (10)$$

In Eqn. (10) $^{18}\text{R}/^{34}\text{R}$ refers to the isotope ratio values of the standard gas and $\Delta\delta^{18}\text{O}$ is the ^{18}O difference between sample and reference SO_2 . Since these values cannot be measured independently in a simple way the expression $^{18}\text{R}/^{34}\text{R}$ is usually computed using the ratio values of VSMOW ($^{18}\text{R} = 0.0020052$, see Table 1) and VCDT ($^{34}\text{R} = 0.0441509$) and $\Delta\delta^{18}\text{O}$ is set to zero. Hence, the correction simply involves multiplying ${}^{66}\delta_{\text{meas}}$ with 1.091 for obtaining $\delta^{34}\text{S}$.

The simplification of Eqn. (10) applies strictly only when the oxygen in both sample and reference SO_2 are isotopically identical ($\Delta\delta^{18}\text{O} = 0$) and equal to VSMOW and the sulfur is isotopically equal to VCDT. Hence, this can only be regarded as a first-order correction. Fortunately, small errors in the adopted ratios do not alter the analytical result significantly. A difference in ^{18}O content in terms of $\delta^{18}\text{O}$, however, translates into an error of the analytical result that is 9% of the $\Delta\delta^{18}\text{O}$ -value.

Due to the 'stickiness' of SO_2 on surfaces, further corrections are necessary and recommended.⁴⁹ A systematic scaling factor of about 1.035 between sulfur measurements using SF_6 and others using SO_2 has been observed by many laboratories. The simplest overall correction is scaling the measured and first-order corrected SO_2 data with known isotopic compositions of reference material prepared together with the unknown samples. For sulfides, IAEA-S-2 (+22.66‰ VCDT) and IAEA-S-3 (−32.30‰ VCDT) provide good references for the scaling exercise. For analyzing barium sulfates, IAEA-SO-5 (+0.49‰ VCDT) and IAEA-SO-6 (−34.18‰ VCDT) will serve the purpose. Please note that the latter two values are provisional.^h

For IRMS techniques, where the SO_2 reference gas comes from a high-pressure cylinder, calculation of data should be made exclusively in terms of ${}^{66}\delta_{\text{meas}}$ -values up to the point where the reference is a sample processed exactly as the other samples (identical treatment again). Only then is the oxygen isotope signature similar enough to use the first-order correction given above.⁵⁰

Further corrections

Drift corrections

During analysis of a series of measurements one often observes a drift of the results as a function of time (or sample number). Drifts can have multiple causes including isotopic change of

the reference gas in the dual inlet system during an analysis sequence, build up of water or other contaminants during analysis, changing conditions of the mass spectrometer for instance following a fresh pump down of the vacuum system, deterioration of ion source conditions and many more. Many of these causes can and should be fixed by changing the conditions of the analysis. However, this can only be accomplished reliably when the overall design of the analytical sequences allows easy detection and provides means for correction of the drifting results. The design rules are simple: A sufficient number of identical samples are interspersed with the analyte samples and used to correct the drift. For an example of drift correction please refer to the spreadsheet in the hydrogen isotope analysis section (Fig. 10).

Linearity corrections

Similar to drift in time is a drift with size, i.e. the measured ratio is a function of the size of the sample (or a reagent). Commonly observed size or linearity effects are the dependence of the measured ratio of the analyte gas on the size of the major ion beam ('pressure effect'), the observed fractionation of hydrogen isotopes as a function of the amount of reduction reagent used, or the dependence of measured $\delta^{18}\text{O}$ -values on temperature during an equilibration experiment. The first and obvious choice is to eliminate the causes of the linearity problems by improving temperature stability or reducing the dwell time of the ions in the high-pressure region of the ion source. This is often successful for reducing the size of the effect. However, a small fraction will almost always remain and thus will have to be corrected for. The general correction strategy is similar to the one described above: A sufficient number of known samples are measured together with the analyte samples. In this case the amount of material is varied in order to detect the size of the required linearity correction.

Correcting cross contamination in dual inlet measurements (η -correction)

Following a switching action of the 'changeover valve' from one gas to the other a delay time ('idle time') is necessary to await complete exchange of the gases in the ion source. Depending on the gas this can be rather short (~ 4 s) or it may take up to several minutes for a complete replacement. Gases like H_2 , N_2 , SF_6 or N_2O have little surface activity and, hence, are pumped away fast whereas SO_2 and CO_2 can take considerably longer to exchange completely. The time depends on the material of the sputtered surfaces, on the sensitivity of the instrument, on the tightness of the ion source design and other parameters. The true δ -value can be calculated from the measured value according to:⁵¹

$$\delta_{\text{true}} = \delta_{\text{meas}} / (1 - 2\eta - \eta\delta_{\text{meas}}/1000) \quad (11)$$

with η describing the cross contamination affecting the background of the subsequent measurement. (Please note that Eqn. (4) in Ref. 51 is cast in absolute terms of δ , not in‰ 'units'). Meijer *et al.*⁵¹ also suggested a correction strategy which comprises (1) experimental determination of the cross contamination parameter η by introducing natural isotope abundance and enriched gas followed by (2) application of Eqn. (11) to the raw analytical results. For CO_2 , the numerical

value for η mostly ranges between 0.001 and 0.01. In some cases (when the ion source is rather tight, the sensitivity is high and the sputtered surface area is large) the η correction can be as large as 1‰ for $\delta^{13}\text{C}$ in CO_2 when the isotopic difference exceeds 40‰.

An alternative and recommended correction for cross contamination is scaling to precisely known (large) differences of reference materials as is routinely done in hydrogen and oxygen isotope ratio analysis by scaling to the SLAP/VSMOW difference. Besides hydrogen and oxygen isotope ratio determination this scale normalization has proven mandatory for sulfur (measured as SO_2) and might also turn out to be required for carbon, in particular when the measured isotopic differences are large and (or) the precision requirements are high.

Reporting of experimental data

Isotope ratio data often enter into a multidimensional puzzle from which further conclusions are drawn. This is the case in almost any carbonate or precipitation study; it is obvious also in CO_2 in air analyses. Sometimes isotope ratio data are valued by other researchers in a way that the original author had not anticipated. As a consequence, isotope ratio data reported in the scientific literature should be presented together with the necessary context that accompanies the pure number. Wherever the source of the scale that was used for referencing experimental values to an international standard may have arisen from, the procedure should and must be described.

For instance, most combustion analyses are calibrated with NBS-22 oil using a published $\delta^{13}\text{C}$ -value of, for example, -29.74‰ on the VPDB scale. This value, however, has been revised a number of times; the latest revision to -29.78‰ happened only recently.¹ Thus, when published data are compared with other data reported vs. VPDB based on a different value or even based on a different material, adjustments can be made, provided the basis of the scale is clear. In addition to the final δ -values, precision ($\equiv 1\sigma$, standard deviation) as well as sample preparation and measurement techniques should accompany the data in order to judge the relative merits of published data.

Similarly, for reporting $\delta^2\text{H}$ -values of substances other than water, it is recommended that the author's measured $\delta^2\text{H}$ of NBS-22 oil, NBS-30 biotite, IAEA-CH-7 polyethylene foil, or other internationally distributed reference material be reported, as appropriate to the analytical method. Please note that normalization to the -428‰ difference of SLAP and VSMOW, as is mandatory for water analysis, requires the availability of new internationally distributed reference materials. The normalization procedure should be stated in the author's report.

For reporting $\delta^{18}\text{O}$ -values of substances other than water or carbonates (including the measurement of pure CO_2 gas), it is recommended that the author's measured $\delta^{18}\text{O}$ of NBS-28 quartz, NBS-30 biotite, NBS-127 barium sulfate, atmospheric oxygen or other internationally distributed reference material be reported, as appropriate to the analytical method. The $\delta^{18}\text{O}$ scale should be normalized such that the $\delta^{18}\text{O}$ of SLAP reference water is -55.5‰ VSMOW exactly, and so stated in the author's report.

CONCLUSIONS

We have described some of the recently developed techniques for referencing stable isotope ratio values in routine operation in our laboratory. The quality control for long-term accuracy is built into the daily cycles. Performance or quality charts help detect errors in high-precision referencing early and provide means for correction. It is common practice in many laboratories to treat sample and reference material in an identical manner. We propose to adopt this practice as the general principle guiding the positioning of measured isotope ratios on international scales.

Acknowledgements

We would like to thank Beate Rothe, Stefan Braeunlich and Heike Geilmann for their excellent performance of most of the analyses described. The students and post-docs in our institute have generously provided samples and weighed or filled them in for measurement. Elizabeth W. Sulzman and Charles B. Douthitt have made helpful comments and corrections to the draft manuscript. We are grateful to T. B. Coplen for his critical and very helpful comments to the first draft of this manuscript in a difficult time and for promoting publication by acting as a sponsor referee for this paper.

REFERENCES

- Murphey BF. *Phys. Rev.* 1947; 72: 834.
- Urey HC. *Science* 1948; 108: 489.
- McKinney CR, McCrea JM, Epstein S, Allen HA and Urey HC. *Rev. Sci. Instrum.* 1950; 21: 724.
- Habfast K. Advanced Isotope Ratio Mass Spectrometry I: Magnetic Isotope Ratio Mass Spectrometers. In I.T. Platzner (ed). *Modern Isotope Ratio Mass Spectrometry*. ISBN 0-471-97416-1. Volume 145 in Chemical Analysis, J. D. Winefordner (series ed.). John Wiley: Chichester, 1997; 11–82.
- Wieser ME and Brand WA. Isotope Ratio Studies Using Mass Spectrometry. In J. C. Lindon, G. E. Tranter and J. L. Holmes (eds). *Encyclopedia of Spectroscopy and Spectrometry*, Academic Press, ISBN 0-12-226680-3, 1999; 1072.
- Brand WA. Mass Spectrometer Hardware for Analyzing Stable Isotope Ratios. In: P. deGroot (ed). *Handbook of Stable Isotope Analytical Techniques*, Elsevier: Amsterdam, accepted for publication 2000.
- Urey HC, Lowenstam HA, Epstein S and McKinney CR. *Bull. Geol. Soc. Am.* 1951; 62: 399.
- Craig H. *Geochim. Cosmochim. Acta* 1953; 3: 53.
- Craig H. *Geochim. Cosmochim. Acta* 1957; 12: 133.
- Gonfiantini R. Advisory Group Meeting on Stable Isotope Reference Samples for Geochemical and Hydrological Investigations, IAEA, Vienna, 1987.
- Coplen TB. *Pure Appl. Chem.* 1994; 66: 273.
- VPDB has a $\delta^{18}\text{O}$ -value of +30.92‰ on the VSMOW scale. λ is the fractionation factor that relates terrestrial ^{17}O and ^{18}O . It is further discussed under ' ^{17}O correction for $\delta^{13}\text{C}$ determination'. A comprehensive review of stable isotope scales and reference materials is the IAEA-TECDOC-825, available from the IAEA in Vienna. Available: <http://www.iaea.org/worldatom/publications/catalogue.html>.
- Zhang Q-L, (Chang TL) and Li WJ. *Chin. Sci. Bull.* 1990; 35: 291.
- Baertschi P. *Earth Planet. Sci. Lett.* 1976; 31: 341.
- Li W, Ni B, Jin D and Zhang Q. *Kexue Tongbao Chin. Sci. Bull., Engl. Trans.* 1988; 33: 1610.
- DeBievre P, Valkiers S, Peiser HS, Taylor PDP and Hansen P. *Metrologia* 1996; 33: 447.
- de Wit JC, van der Straaten CM and Mook WG. *Geostand. Newslett.* 1980; 4: 33.
- Ding T, Bai R, Li Y, Wan D, Zou X and Zhang Q. *Science in China (Series D)* 1999; 42: 45.
- Epstein S, Buchsbaum R, Lowenstam HA and Urey HC. *Bull. Geol. Soc. Am.* 1951; 62: 417.
- Epstein S and Mayeda T. *Geochim. Cosmochim. Acta* 1953; 4: 213.
- McCrea JM. *J. Chem. Phys.* 1950; 18: 849.
- Coplen TB, Kendall C and Hopple J. *Nature* 1983; 302: 236. This paper recommends the use of +30.91‰ for VPDB vs. VSMOW. However, NBS-19, the carbonate which now defines the VPDB scale, was measured and reported as -2.19‰ vs. PDB. Correction of this value to -2.2‰ leads to the revised value of +30.92‰ vs. VPDB.
- Friedman I and O'Neil JR. *US Geological Survey Professional Paper* (1977); 440-KK.
- Junk G and Svec H. *Geochim. Cosmochim. Acta* 1958; 14: 234.
- Mariotti A. *Nature* 1983; 303: 685.
- Coplen TB and Krouse HR. *Nature* 1998; 392: 32.
- Beaudoin G, Taylor BE, Rumble III D and Thiemens M. *Geochim. Cosmochim. Acta* 1994; 58: 4253.
- MacNamara J and Thode HG. *Phys. Rev.* 1950; 78: 307.
- Halsted RE and Nier AO. *Rev. Sci. Instrum.* 1950; 21: 1019.
- Available: http://www.cio.phys.rug.nl/HTML-docs/Ver-slag/97/report_95-97.htm.
- Brand WA. *J. Mass Spectrom.* 1996; 31: 225.
- Matthews DE and Hayes JM. *Anal. Chem.* 1978; 50: 1465.
- Hayes JM, Freeman KH, Popp BN and Hoham CH. *Org. Geochem.* 1990; 16: 1115.
- Merritt DA, Brand WA and Hayes JM. *Org. Geochem.* 1994; 21: 573.
- Werner RA, Bruch BA and Brand WA. *Rapid Commun. Mass Spectrom.* 1999; 13: 1237.
- Kornel BE, Werner RA and Gehre M. *Rapid Commun. Mass Spectrom.* 1999; 13: 1248.
- Spreadsheets are the perfect tool for establishing the data analysis schemes and procedures. They can, however, become cumbersome with time, in particular when information from the past must be retrieved from a large pile of data and the criteria for the retrieval have changed. Once the procedures in the laboratory have become stable and mature the use of spreadsheet should therefore be replaced by a database system that handles the sample flow and reporting as well as the retrieval of the results. An example of such a LIMS system has been developed by T. B. Coplen, USGS, Reston, and is described in T. B. Coplen, A Guide for the Laboratory Information Management System (LIMS) for Light Stable Isotopes, Versions 7 and 8, US Geological Survey, Open-File Report 00-345, 121 pp.
- DaCosta G and Steele P. *Tenth WMO Meeting of Experts on Carbon Dioxide Concentration and Related Tracer Measurement Techniques*, Stockholm, 1999.
- Brand WA, Avak H, Seedorf R, Hofmann D and Conradi T. *Isot. Environ. Health Stud.* 1996; 32: 263.
- Coplen TB. *Chem. Geol. (Isot. Geosci. Sect.)* 1988; 72: 293.
- See for instance <http://www.cmdl.noaa.gov/ccgg/figures/isotopes.gif>.
- Brand WA and Werner RA. in preparation.
- Matsuhisa Y, Goldsmith JR and Clayton RN. *Geochim. Cosmochim. Acta* 1978; 42: 173.
- Meijer HAJ and Li WJ. *Isot. Environ. Health Stud.* 1998; 34: 349.
- Miller MF, Franchi IA, Sexton AS and Pillinger CT. *Rapid Commun. Mass Spectrom.* 1999; 13: 1211.
- Allison CE, Francey RJ and Meijer HAJ. *Recommendations for the Reporting of Stable Isotope Measurements of Carbon and Oxygen in CO₂ gas [1]*. In IAEA-TECDOC-825, IAEA, Vienna, 1995; 155–162.
- Santrock J, Studley SA and Hayes JM. *Anal. Chem.* 1985; 57: 1444.
- Gonfiantini R, Stichler W and Rozanski K. *Standards and Intercomparison Materials Distributed by the IAEA for Stable Isotope Measurements*. In IAEA-TECDOC-825, IAEA, Vienna, 1995; 13–29.
- Rees CE. *Geochim. Cosmochim. Acta* 1978; 42: 383.
- Giesemann A, Jäger HJ, Norman AL, Krouse HR and Brand WA. *Anal. Chem.* 1994; 66: 2816.
- Meijer HAJ, Neubert REM and Visser GH. *Int. J. Mass Spectrom.* 2000; 198: 45; Rama Rao VVK, Bindal SN. *Int. J. Mass Spectrom.* 2001; 206: 177.
- Kim SA and O'Neil JR. *Geochim. Cosmochim. Acta* 1997; 61: 3461.

HIGH-PRECISION CONTINUOUS-FLOW ISOTOPE RATIO MASS SPECTROMETRY

**J. Thomas Brenna, Thomas N. Corso,* Herbert J. Tobias,[†]
Richard J. Caimi**

Division of Nutritional Sciences, Cornell University, Ithaca, New York 14853

Received May 1997; accepted 26 November 1997

| | |
|---|-----|
| I. High-Precision Mass Spectrometry..... | 228 |
| A. Isotope Ratio Mass Spectrometry (IRMS) | 229 |
| 1. High-Precision | 229 |
| 2. Ion Source and Dual Inlet | 229 |
| 3. Analyzer | 230 |
| 4. Detection and Data Acquisition | 231 |
| 5. Isotopic Standards | 232 |
| II. Dual Inlet Analysis | 232 |
| A. Off-Line Sample Preparation for Organics | 232 |
| B. Water Analysis | 232 |
| III. Continuous-Flow (CF) IRMS | 233 |
| A. Bulk Samples: Elemental Analyzer | 234 |
| B. Trace Gases | 235 |
| C. Breath Tests | 235 |
| IV. Compound-Specific Isotope Analysis (CSIA)..... | 236 |
| A. Gas Chromatography (GC) and Carbon | 236 |
| B. CSIA/CF Data Analysis | 238 |
| C. GC and Nitrogen | 239 |
| D. General CF Analysis of Hydrogen | 240 |
| E. Water Analysis | 241 |
| F. CF Analysis of Hydrogen: Correction Procedures | 241 |
| G. CSIA for Hydrogen | 242 |
| H. Liquid Chromatography-Combustion (LCC)-IRMS | 243 |
| 1. Moving Transports | 243 |
| 2. LC/Chemical Reaction Interface (CRI)/IRMS | 245 |
| V. Position-Specific Isotope Analysis (PSIA)..... | 245 |
| VI. Notation and Elementary Calculations | 248 |
| A. Delta Notation | 248 |
| B. Atom Fraction (Percent) Notation | 249 |
| C. Mass Balance | 249 |
| D. Tracer Studies Using High-Precision CSIA | 250 |
| E. Mass Balance: Pool Mixing | 251 |
| F. Dilution Space | 253 |
| VII. Conclusions | 253 |
| References | 254 |

Correspondence to: J. Thomas Brenna, Phone: (607) 255-9182, Fax: (607) 255-1033, JTB4@CORNELL.EDU.

* Present address: Advanced Bioanalytical Systems, Inc, 15 Catherwood Rd, Ithaca, NY 14850.

[†] Present address: Department of Chemistry, University of California, Riverside, CA.

Contract grant sponsors: National Institute of General Medical Sciences of NIH, Chevron Petroleum Technology Co., and the USDA-CSREES. Contract grant nos.: NIH grant GM49209 and Training grant DK07158.

Although high-precision isotope determinations are routine in many areas of natural science, the instrument principles for their measurements have remained remarkably unchanged for four decades. The introduction of continuous-flow techniques to isotope ratio mass spectrometry (IRMS) instrumentation has precipitated a rapid expansion in capabilities for high-precision measurement of C, N, O, S, and H isotopes in the 1990s. Elemental analyzers, based on the flash combustion of solid organic samples, are interfaced to IRMS to facilitate routine C and N isotopic analysis of unprocessed samples. Gas/liquid equilibrators have automated O and H isotopic analysis of water in untreated aqueous fluids as complex as urine. Automated cryogenic concentrators permit analysis at part-per-million concentrations in environmental samples. Capillary gas chromatography interfaced to IRMS via on-line microchemistry facilitates compound-specific isotope analysis (CSIA) for purified organic analytes of 1 nmol of C, N, or O. GC-based CSIA for hydrogen and liquid chromatography-based interfaces to IRMS have both been demonstrated, and continuing progress promises to bring these advances to routine use. Automated position-specific isotope analysis (PSIA) using noncatalytic pyrolysis has been shown to produce fragments without appreciable carbon scrambling or major isotopic fractionation, and shows great promise for intramolecular isotope ratio analysis. Finally, IRMS notation and useful elementary isotopic relationships derived from the fundamental mass balance equation are presented. © 1998 John Wiley & Sons, Inc., Mass Spectrom Rev 16, 227–258, 1997

I. HIGH-PRECISION MASS SPECTROMETRY

Mass spectrometry (MS) is best known among chemists as one of the most important analytical techniques for the characterization of molecular structure and amount, elemental composition and spatial arrangement (Burlingame, Boyd, & Gaskell, 1996). Remarkably, among a large cross-section of biomedical, biological, and other natural scientists it is better known as a tool to reveal the origin, genesis, or state of complex systems through high-precision isotope ratio measurements. Careful determination of isotope abundances reveal this other dimension of matter, which often is not available from structural or quantitative studies.

Soon after the “natural” abundances of the elements were determined, variations linked to material sources were noticed, and systematics were described, starting with Nier and Gulbransen’s 1939 observation that the concentration of ^{13}C is greater in carbonates than in organic carbon (Nier & Gulbransen, 1939). Isotope variation due to natural processes is now known throughout natural science and has provided important data on phenomena as diverse as respiration in trees (Dawson & Ehleringer, 1993), dietary studies of ancient humans (“paleodietary reconstruction”) (Stott & Evershed, 1996), and contemporary animals (e.g., seal lion foodwebs (Hobson et al.,

1997), nitrification rates in forests (Starle & Hart, 1997), biodegradation (Aggarwal et al., 1997), and the climate history of the earth (Schoell et al., 1994). In ecology and biogeochemistry alone, two volumes (Ehleringer & Rundel, 1988; Ehleringer, Hall, & Farquhar, 1993) and at least one meeting (Griffiths, 1996) devoted to applications of high-precision isotope measurements have appeared in the last few years. Isotope ratio MS (IRMS) has penetrated areas of biological science to a greater degree than organic mass spectrometry. In addition to studies of natural variability, high-precision isotope measurements facilitate a number of tracer techniques in the biomedical sciences, including the doubly labeled water method for energy expenditure (Schoeller et al., 1986), protein turnover studies (Chapman et al., 1990), fat metabolism (Brenna, 1997), and a range of breath tests with the potential to revolutionize clinical diagnostic testing (Ghoos, Rutgeerts, & van Trappen, 1995).

The early history of MS was dominated by physical measurements to establish the nominal, terrestrial isotopic abundances of the elements. The early parabola method resulted in the discovery of two forms of Ne in 1911 by J. J. Thompson, with the recognition in 1919 that these ions were isotopes, using the first mass spectrograph (Aston, 1942). The latter event is arguably the first gas isotope ratio mass spectrometry (IRMS) experiment. Determination of the isotopic abundances of the elements was an application so dominant that one of its most productive participants, F. W. Aston, is said to have predicted the demise of MS as a field of research once the isotopic abundances of all the elements had been determined (Svec, 1985). It was, however, difficult to foresee the utility of high-precision isotope ratios as clues to a sample’s origin or history, or the importance of isotopes as artificial tracers. Even in the 1930s, before the isotopic abundances of all the elements had been established, the MS analysis of stable isotopes as tracers was yielding important breakthroughs in the bio-sciences (Clarke, 1948).

Natural abundance applications for the most part require the high-precision techniques introduced in the late 1940s; these techniques dominated IRMS for four decades. They focused initially on the analysis of bulk environmental materials of high chemical complexity such as plant or petroleum reduced to simple analysis by combustion to CO_2 or conversion to N_2 or H_2 . Analysis of chemically pure materials was much more common for compounds that occur in relatively pure form in nature such as water, because off-line separation is a time-consuming affair fraught with the risk of contamination and subtle isotopic fractionation. Much less common is the even more cumbersome analysis of intramolecular isotope ratios, which require the manual isolation of individual positions in a molecule following chemical isolation, all in an isotopically representative and uncontaminated form.

Fundamental changes in inlet systems introduced to multicollector IRMS in the 1980s, permitting detailed analysis of complex mixtures, are now fueling a revolution in IRMS, in the same way that analogous innovations did for organic MS in the 1950s and 1960s (Gohlke & McLafferty, 1993; McLafferty, 1997). High-precision "compound-specific isotope analysis" (CSIA), coupling GC separation to IRMS, ushered in dramatic improvements in sensitivity and analysis speed for chemically pure analytes and complex mixtures. Referred to generically as continuous flow (CF) instruments, their commercial appearance around 1990 precipitated a dramatic increase in research on instrumentation and methods in high-precision isotope ratio mass spectrometry. Recent advances also offer the promise of automated high-precision position-specific isotope analysis (PSIA), a field that has seen remarkably little experimental advancement in spite of the ubiquity of intramolecular isotopic variability in nature.

It is our purpose to review the current status of CF-IRMS instrumentation. After a brief review of classical IRMS instrumentation, we focus on CF introduction systems, which facilitate rapid, bulk, compound-specific, and position-specific isotope analysis. We close with a review of some basic calculations that are essential for the manipulation of high-precision isotope ratio results.

A. Isotope Ratio Mass Spectrometry (IRMS)

1. High-Precision

All mass spectrometers can measure isotope abundances in one way or another. The distinguishing characteristic of isotope ratio mass spectrometers (IRMS) is a measurement at *high-precision*, which is defined generally as a standard deviation in the range of 4–6 significant figures. High-precision isotope measurements by MS are possible for all polybaric elements of the periodic table by one of three instrument types, generally referred to as gas IRMS, *thermal ionization MS* (TIMS) for metals, and *static gas* or *noble gas* instruments. The designation IRMS usually refers to the gas-source machine that is dedicated to the analysis of light elements, and frequently the term *gas IRMS* (GIRMS) is used to distinguish it from TIMS. IRMS operates on different principles than TIMS or noble gas machines, which are outside the scope of this review. A recent review documenting commercial vendors for all these instruments has appeared (Newman, 1996).

High-precision is accomplished at the expense of flexibility. IRMS instruments accept the analyte in the form of one of a limited number of gases, which must be isotopically representative of the original sample. $^{13}\text{C}/^{12}\text{C}$, $^2\text{H}/^1\text{H}$, $^{15}\text{N}/^{14}\text{N}$, $^{18}\text{O}/^{16}\text{O}$, and $^{34}\text{S}/^{32}\text{S}$ analyses are performed through analysis of the pure gases of CO_2 , H_2 , N_2 , N_2O , NO , O_2 , CO , and SO_2 or SF_6 ; for CF applications, these

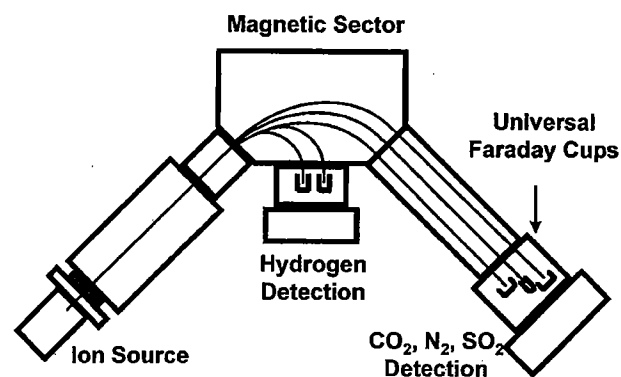


FIGURE 1. Diagram of a generic isotope ratio mass spectrometer, not including inlets. A tight electron impact ion source efficiently generates positive ions that are mass-analyzed by a single magnetic sector. Multiple Faraday cups permit simultaneous and continuous monitoring of the analyte major isotopomer masses in a split flight tube for hydrogen or for other gases. Universal collectors with a central narrow cup and two outer wider cups are used for capturing higher mass gases such as CO_2 and N_2 .

gases will usually be in He carrier gas. These gases are the only ones that are admitted to the ion source of the IRMS.

The mass spectrometer design was developed by A. O. Nier in the 1940s and is the basis of modern instruments (Nier, 1940; 1947; Murphey, 1947; McKenney et al., 1950). A diagram of the basic instrument is presented in Fig. 1, which shows the ion source, single magnetic sector, and multiple collectors for the various isotopomers. Several features distinguish the components of the IRMS instrument from mass spectrometers used for structural studies, which we refer to as *organic MS*. These features are driven by the high-precision aspect of IRMS that requires that the detection probability be maximized to optimize counting statistics. In effect, the flexibility of organic MS is sacrificed to tailor the IRMS to very limited but high-performance measurements. An excellent detailed discussion of the design and theoretical performance of the ion source, ion optics, mass analyzer, and collectors for an IRMS is available (Prosser, 1993).

2. Ion Source and Dual Inlet

The IRMS employs an electron impact (EI) ion source specifically tailored to maximize ionization probability. Organic MS EI ion sources are designed so that the analyte stream passes through the electron beam once, where analyte molecules are either ionized or pass out of the source and are pumped away. Ionized molecules are rapidly accelerated into the analyzer section before collisions can take place. Reactive collisions that result in ions of weights greater than the analyte molecule would complicate EI

mass spectra greatly, because the highest mass ion would no longer be the putative molecular ion, and spectra would be dependent on ion source pressure. In addition, ion sources would require frequent cleaning, because reactive higher molecular weight products are more prone to condensation than lower molecular weights. Effectively, organic EI sources are nearly collisionless and are referred to as "open" sources.

The IRMS ion source is referred to as a "tight" source, because the goal is to contain analyte molecules until they are ionized to maximize ionization probability. Analyte gas molecules undergo many collisions with each other and the interior source walls prior to electron impact ionization, and nascent ions undergo collisions with neutral analyte molecules. In this regard, the IRMS EI source is much more like an organic chemical ionization (CI) source, which depends on collisions to facilitate proton exchange or other processes. Ion-molecule reactions do occur for IRMS gases when there is a source of hydrogen in the ion source. The most common source is the $H_2-H_2^+$ reaction that results in the formation of H_3^+ , which is an important interference in hydrogen analysis. The high collision rate of the EI source is one of the principal reasons why the analyte must be converted to a low molecular weight, relatively unreactive gas, and why hydrogen must be excluded whenever possible.

The ion source produces a 70-eV electron beam, collimated by small source magnets, for the EI ionization of gas molecules. The tight source produces high ion efficiencies of about 1 ion per 10^3 molecules that enter the source. This efficiency compares to that of about 1 ion in 10^6 molecules for the conventional open organic ion sources that are used for structural chemical analysis. This sensitivity advantage is usually even greater on a per mol analyte basis, because analyte molecules must be converted to an acceptable analysis gas. For instance, each molecule of methyl stearate introduced to an organic MS has a single chance for ionization. IRMS analysis requires conversion of the 19-carbon methyl stearate molecule into 19 molecules of CO_2 , each of which has an independent probability of ionization in the IRMS. Differences in ionization energies and cross-sections, and extent of fragmentation exist between large organic molecules and CO_2 . Nevertheless, an advantage of an order of magnitude in sensitivity can be expected in our example.

From this example, and in contrast to organic MS, it can be seen that it is inappropriate to quote "detection limits" for IRMS in the classical analytical chemical sense. Rather, the minimum amount of analyte required for analysis is appropriately expressed as mol of analyte element (e.g., C, N, etc.) required to yield a specified precision. For some elements, such as C, the *weight* of analyte is closely related to the molar mass; thus, it is

simplest to express lower analytical limits in terms of analyte mass. For example, the lower limits of analysis for an amino acid of molecular weight 100 and for a protein of molecular weight 100,000 are both approximately 10 ng, or about 1 nmol C.

Collisions in the ion source interact in a complex way to produce a well-known nonlinear dependence of ion current ratios with source pressure. The electron beam-collimating magnets exert a mass-dependent influence on the flight path of nascent ions as they are accelerated out of the source. The precise region within the source from which ions escape is related to source pressure; hence, the degree of flight path influence is related to source pressure (Kirshenbaum, 1951). It is easy to show that a plot of ion current ratio vs. m/z 44 intensity, I_{45}/I_{44} vs. I_{44} for carbon analysis, has a linear dependence on pressure that can be essentially eliminated by using He carrier gas to buffer analyte concentration changes (Tobias & Brenna, 1996). In the absence of a buffer gas, high-precision and accuracy demand that sample and standard be analyzed at the same pressure, which should be as constant as practical.

The dual-inlet system was first introduced in 1947 by Murphey (1947) for the analysis of gas diffusion rates, and it was soon incorporated into the classic analytical IRMS instrument design of McKinney et al. (1950). That design is a landmark usually taken to be the birth of high-precision IRMS. Modern dual-inlet systems are computer-controlled with adjustable welded-metal bellows in which sample and standard gas are stored. The pressures of the bellows are matched so that both gases enter the ion source at the same flow rate and pressure. In addition, the capillary used to feed the gases into the source is crimped to set flow resistance and permits flow rates to be matched. Through the use of automated valves, the sample and standard gases can be alternately delivered into the IRMS while the other volume is depleted and vented to waste at a matched rate to maintain equivalent pressures in both bellows.

3. Analyzer

Mass resolution is sacrificed to optimize stability and transmission, which are the two most important features for IRMS mass analysis. The IRMS analyzer section starts with an entrance slit that is typically of fixed width and gives a working resolution of about $m/\Delta m = 100$, which is sufficient to separate the masses of interest while maintaining maximum transmission. Ions are extracted and accelerated out of the ion source at 2–10 keV, collimated into a beam by electrostatic lenses, and deflected electrostatically into a magnet with a field of around 0.75 tesla. The analyzer is always a single magnetic sector, which is sometimes a permanent magnet, requiring that mass adjustments be made by adjusting the accelerating voltage.

A uniform magnetic field is required to produce multiple well-defined ion beams emerging toward the detectors. High mass resolution is not required and, thus, electric sectors are uncommon.

In the particular case of CF analysis of HD entering the ion source in a stream of He, high-performance mass analysis is desirable. High-abundance sensitivity is useful to separate the tail of the very large He peak at m/z 4 from the adjacent HD peak at m/z 3. One manufacturer introduced such an instrument in 1995 that can comfortably make this analysis; this instrument will be detailed in the hydrogen section below.

4. Detection and Data Acquisition

The majority of organic MS instruments employ electron multipliers (EM) for ion detection, with the most prominent exception being trap-type instruments such as Ion Cyclotron Resonance (Marshall & Verdun, 1990) and a recently reported advance in rf ion traps (Soni et al., 1996). IRMS instruments are probably the only analytical gas source mass spectrometers in commercial production at this writing that employ faraday cups (FC) for ion detection. Multiple cups are in routine use, one for each ion beam of interest, to maximize dwell times and eliminate the need for peak-jumping. Simultaneous measurement also improves precision dramatically, because it eliminates variability in analytical conditions that cause correlated changes in signal intensity such as filament brightness. Deep cups with secondary electron suppressers capture analyte ions and the secondary electrons emitted upon impact of high-energy ions onto the metal surface. Dedicated amplification electronics are required for each mass channel. FCs are generally less sensitive than EMs, but are superior for IRMS because (1) the high count rates required for high-precision place IRMS signals in the normal detection range for FCs, but would rapidly damage EMs; (2) FCs linearly detect very large ion currents without gain adjustments; (3) they are highly stable; and (4) with the low atomic weight elements used in IRMS, they have very long lifetimes that are measured in decades.

Amplification of signal at the FC is accomplished by dedicated circuits mounted in close physical proximity to the detectors. The feedback amplifiers are usually highly shielded, sometimes held under vacuum, and are made with high performance resistors to yield amplification factors corresponding to the approximate isotopic abundance in nature. For example, the feedback resistors for H₂ and HD are in the nominal ratio 1:1000 and, when digitized, produce signals that have heights about 1:0.3, corresponding to the 150 parts-per-million abundance of D compared to H, after correcting for the dual probability of D appearance in HD. Matching of signals is critical for the high-precision comparisons central to IRMS data analysis in

dual inlet analysis, because there is no He carrier gas to equalize pressures. It is much less important for continuously varying analyte signal in CF analysis, except for H analysis. Poor signal matching is the major factor why signals that diverge significantly from natural abundance (in tracer experiments, for example) commonly yield poor precision and accuracy.

The dual-inlet data stream is extraordinarily straightforward. A sample from each inlet is admitted to the IRMS, the signal is allowed to stabilize, and data are collected at a plateau level. After a preset measurement time, inlets are switched and the cycle is repeated several times. Ion current ratios are converted to isotope ratios using the delta notation suggested by Urey in 1948 (Hayes, 1983), shown as follows for the specific case of carbon:

$$\delta 45 = \left(\frac{R_{SPL} - R_{STD}}{R_{STD}} \right) \times 1000 = \left(\frac{R_{SPL}}{R_{STD}} - 1 \right) \times 1000;$$

$$R_x = \frac{[45]}{[44]}, \quad (1)$$

where [45]/[44] is the ion current ratio, and $\delta 45$ includes contributions from both ¹³C- and ¹⁷O-substituted CO₂. For carbon, an expression for ¹³C/¹²C is normally desired, and correction procedures have been devised to use the mass 46 signal, which is primarily representative of the ¹²C¹⁸O¹⁶O species, and the correlation in nature between ¹⁸O and ¹⁷O, to convert $\delta 45$ to $\delta^{13}\text{C}$, relative to the working standard (Craig, 1957; Santrock, Stanley, & Hayes, 1985). A final adjustment converts the sample isotope ratio into terms relative to the international standard Pee Dee Belemnite, in parts "per mil" or "per mille" (‰). This notation has two advantages. First, it emphasizes that the high-precision component of the measurement is relative to a standard, rather than an absolute measure. Second, isotopic fractionation in nature is subtle, yielding changes in the fourth significant figure and beyond; the $\delta^{13}\text{C}$ notation eliminates the leading digits common among nearly all but artificially enriched samples and focuses on changing digits. We return to isotope notation later.

Analogous calculations are performed for CF data, except that the ion current ratios are normally derived from peak areas rather than plateau regions. The CF data stream consists of a time array and two or three signal arrays, which are most analogous to selected ion monitoring chromatographic traces in organic MS. Signals must be digitized to about 20 bits (>10⁶) to retain sufficient precision, and suitable precisions are available on modest cost chromatography data acquisition boards designed to record data from a flame ionization detector (FID), and offer effective duty cycles of greater than 95%.

5. Isotopic Standards

We briefly mention the existence of international isotopic standards for the IRMS elements because more complete discussions can be found elsewhere (Ehleringer & Rundel, 1988). Standards are often calibrated by several laboratories in "round-robin" or "ring" tests, where many laboratories blindly analyze carefully prepared aliquots (for example, see (Schoell & Faber, 1983)). A 1987 *Mass Spectrometry Reviews* article lists IRMS international standards for H, C, N, and O complete with references (Table I of (Hachey et al., 1987)). Many of the primary standards are available today and can be obtained by individual laboratories for use as calibrants for day-to-day working standards.

For C, N, O, and S, a single international standard is normally adequate for isotope ratio calibration of naturally occurring samples. Hydrogen is somewhat unique in its requirement for standards, because the range of naturally occurring D/H concentrations is so large that calibration with a single standard can lead to significant errors for samples of isotope ratio that are very different than the single standard. In 1978, Gonfiantini (1978) proposed a normalization of hydrogen and oxygen isotopic data by using δD and $\delta^{18}O = 0\text{‰}$ for the reference water *Vienna-Standard Mean Ocean Water* (V-SMOW), and $\delta D = -428\text{‰}$ and $\delta^{18}O = -55.5\text{‰}$ for the reference water *Standard Light Antarctic Precipitation* (SLAP), creating the VSMOW-SLAP scale. Individual instruments analyze VSMOW and SLAP, and assign the quoted values to the SLAP data, where the subscript represents normalization to the VSMOW-SLAP scale (Coplen, 1988; 1996). This calibration is known in the isotope ratio field as a "stretching or shrinking" of the isotope ratio scale, so that a second reference material is set to a defined δD value relative to the first reference material. In essence, this procedure is a two-point calibration of ion-current ratio to isotope ratio.

II. DUAL INLET ANALYSIS

A. Off-Line Sample Preparation for Organics

We briefly mention off-line sample preparation for dual inlet analysis. A more detailed discussion is available in previous *Mass Spectrometry Reviews* articles on IRMS (Hatchey et al., 1987; Wong & Klein, 1986). Typically, off-line preparation involves multiple steps on custom-designed vacuum lines equipped with high-vacuum and sample-compression pumps, concentrators using cryogenic or chemical traps, reactions in furnaces using catalysts or true reagents, and microdistillation steps. Contamination and isotopic fractionation are possible at any step,

and, in general, manual off-line methods are slow and tedious, usually requiring large sample amounts, and often requiring considerable operator skill and stamina to produce useful results.

A purified organic sample can be combusted to CO_2 and H_2O using cupric oxide (Wong & Klein, 1986), either by a static method (Sofer, 1980; Schimmelmann & DeNiro, 1993), where the sample is oxidized in an evacuated sealed tube (Boutton et al., 1983) containing copper oxide at $>700^\circ C$; or by a dynamic method (Craig, 1953), where combustion is performed in a vacuum line in the presence of excess oxygen. CO_2 can be analyzed directly or cryogenically separated from the water, and analyzed for its $^{13}C/^{12}C$ ratio using dual inlet analysis. For D/H measurement, the purified water can be reduced by zinc at $>450^\circ C$ in a sealed tube (Coleman et al., 1982) or dynamically reduced over hot uranium (Frazer, 1962) or zinc (Friedman, 1953) with subsequent dual inlet analysis. However, problems of isotopic fractionation and sample memory effects (Wong, Cabrera, & Klein, 1984) result from the difficulty with working with water vapor. The direct conversion of organic hydrogen to hydrogen gas utilizing pyrolysis at $>900^\circ C$ (Sofer, 1986; Sofer & Schiefelbein, 1986) can eliminate the need for handling water vapor in a vacuum line.

Nitrogen samples can be converted to ammonium by Kjeldahl digestion for total nitrogen analysis, followed by ammonium oxidation to nitrogen gas by the Rittenberg technique (Sprinson & Rittenberg, 1949) for $^{15}N/^{14}N$ isotopic analysis (San Pietro, 1957). A superior alternative to this technique is Dumas combustion (Steyermark, 1961), where organic matter is converted to nitrogen in a single-step process (Wada et al., 1977; Pella, 1973). However, the use of an apparatus that is usually employed for nitrogen isotope analysis can lead to inaccuracies through contaminants such as CO , small hydrocarbons, and nitrogen oxides, which produce isobaric interferences with the masses of interest (m/z 28, 29, 30). A combustion procedure using calcium oxide for total removal of CO_2 and H_2O has been developed and helps avoid these problems (Kendall & Grim, 1990). All of these off-line chemical techniques for C, H, and N isotopic analysis are very laborious and time-consuming. Usually, the determination of $^{18}O/^{16}O$ ratio in organic matter is performed by mass spectrometric analysis of CO_2 . Nickel is often used for organic combustion to CO_2 (Brenninkmeijer & Mook, 1981) or samples can be prepared using the $HgCl_2$ pyrolysis method at $550^\circ C$ described by Hayes (Schimmelmann & DeNiro, 1993; Wedeking & Hayes, 1983).

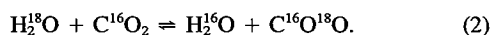
B. Water Analysis

The isotopic analysis of D/H and $^{18}O/^{16}O$ in water is a special application of particular interest to nutritional and

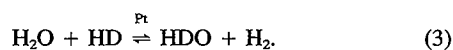
environmental studies. A useful review of off-line sample preparation for D/H and $^{18}\text{O}/^{16}\text{O}$ isotopic analysis of water and organic samples appeared in a previous *Mass Spectrometry Reviews* article (Wong & Klein, 1986). The inconvenience of the off-line static sample preparation for water analysis using the chemical reduction routine particularly for D/H measurements can be reduced by the use of batch processing (Kendall & Coplen, 1985) but off-line preparation continues to demand high operator skill and stamina to produce acceptable results. Electrolytic conversion of water to molecular O_2 and H_2 (Kirshenbaum, 1996) for subsequent isotopic analysis is problematic due to severe fractionation without complete conversion. The procedure is time-consuming and requires large sample quantities (Wong & Klein, 1986), but can be brought down to 20 min per sample with a microelectrolyzer (Bocek, Deml, & Tesarik, 1973).

Direct mass spectrometry of water has been attempted for O isotopic measurements with limited success. An on-line, static system commercially available in the 1980s allowed the simultaneous determination of D/H and $^{18}\text{O}/^{16}\text{O}$ in aqueous samples (Wong, Cabrera, & Klein, 1984). This system consisted of an on-line uranium furnace, for conversion of H_2O to hydrogen gas, preceding a dual mass spectrometer for HD/ H_2 and $\text{H}_2^{18}\text{O}/\text{H}_2^{16}\text{O}$ detection. This system suffered from severe memory effects and required a washout procedure consisting of more than six injections of a sample between analytes of different enrichment before accurate data could be obtained.

An alternative that requires very little operator interaction is the equilibration techniques that have been used for $^{18}\text{O}/^{16}\text{O}$ for decades, but only recently have been practically applied to D/H. The $^{18}\text{O}/^{16}\text{O}$ ratio is measured from CO_2 , with known O isotopic composition, equilibrated with the water sample at room temperature (Cohn & Urey, 1938):



The D/H ratio is measured from H_2 , with known H isotopic composition (Horita et al., 1989; Coplen, Wildman, & Chen, 1991), equilibrated with the water sample:



The use of these chemical equilibration methods for water analysis reduces sample preparation requirements compared to chemical reduction. The isotopic composition of hydrogen in equilibrium with water can be very temperature-dependent (6‰/°C). To achieve reproducible results using a fast equilibrating Pt catalyst, extremely high temperature stability is necessary (± 0.1 – 0.05°C). Slow equilibrating Pt reagents are the only viable solution yet demonstrated to mitigate the effects of this sensitivity (Scrim-

geour et al., 1993). In addition, these methods produce hydrogen gas with a severely depleted D/H ratio, which is taken into account during the calibration procedure. Because precision depends on the number of counts of the less abundant isotope, this depletion requires additional measurement time to yield precisions that are equivalent to samples that are less fractionated during their preparation.

SO_2 is the most common gas employed for S isotopic analysis; however, SF_6 has been used and has the advantage that F is monoisotopic so that there are no isobaric interferences with S isotopes. Off-line conversion of sample S to SO_2 requires several chemical steps in which the sample is first converted to BaSO_4 by acid digestion/precipitation followed by reduction to H_2S and a multistep conversion to SO_2 , as outlined elsewhere (Giesemann et al., 1994). These procedures typically require four hours per sample to generate isotopically representative SO_2 .

III. CONTINUOUS-FLOW (CF) IRMS

The seeds for CF-IRMS derive from the early days of organic MS, when it was shown that separation coupled to analysis by MS yielded results of unparalleled sensitivity, speed, and usefulness. Interfaces between gas chromatographs (GC) and MS, and later other separation devices and MS, focused on the continuous delivery of an analyte with intact structure to the MS in a gas phase form for structural analysis.

The earliest articles to demonstrate purposeful on-line chemical alteration prior to MS intending to simplify isotopic analysis appeared in the late 1970s, starting with Sano and coworkers (Sano et al., 1976), who coupled a GC to an organic MS by means of a combustion furnace. They combusted their sample to CO_2 and set the mass spectrometer to alternate between masses 44 and 45, thereby monitoring the carbon isotope ratio. Their samples were metabolites of a ^{13}C -labeled drug, from which they identified labeled peaks as indicative of metabolic activity. Soon afterward, Matthews and Hayes reported a GC-combustion-MS system based on a single collector organic MS (Matthews & Hayes, 1978). The GC effluent was combusted, and dried with a semipermeable membrane, NafionTM, which passes water and retains CO_2 . The dried effluent was admitted to a single collector MS that was optimized for isotopic analysis and that determined C and N isotope ratios. The system was the first to yield sufficient precision to detect isotope ratio variability in natural sources in compounds separated on-line. Four years later, Markey and Abramson reported a similar approach with on-line chemistry called the chemical reaction interface (CRI) MS, which is based on the processing of a GC effluent in a microwave cavity to yield small molecules for isotopic analysis at low precision for biotracer experiments

(Markey & Abramson, 1982). All of these systems rely on a carrier gas to transport the analyte through a stage of on-line chemistry for conversion to a form acceptable by the MS. This process is one of the fundamental principles underlying CF-IRMS.

A. Bulk Samples: Elemental Analyzer

With the wide use of stable isotopes for bulk analysis in fields such as geochemistry, ecology, biomedicine, and nutrition, a great demand exists for the rapid analysis of large numbers of solid samples. Time-consuming conventional preparation techniques are difficult to automate, and they limit the number of samples that can be analyzed.

A classical method for elemental analysis is based on the flash combustion of samples in a furnace followed by quantitative analysis of the resulting gases (Holt & Hughes, 1955). A commercially available approach to these analyses is automated flash combustion followed by GC separation of CO_2 and N_2 and nondestructive detection using a thermal conductivity detector, with the effluent stream ultimately vented to waste.

In 1983, Preston and Owens (Preston & Owens, 1983) demonstrated the first CF interface to a multi-(dual) collector IRMS for the bulk analysis of nitrogen from solid samples. Precisions of about $\text{SD}(\delta^{15}\text{N}) = 0.7\text{‰}$ were obtained for $3.5 \mu\text{mol}$ aliquots of N_2 derived from urea in the CF mode after flash combustion and separation in a GC equipped with a gas separation column. These authors observed a degradation in the precision due to ion source nonlinearities when analyzing samples of varying size, as is now observed in demanding CF analyses. This system was the first high-precision multicollector CF-IRMS. Two years later, these authors extended the instrument to the analysis of C with similar reproducibility (Preston & Owens, 1985).

Modern commercially available elemental analyzers provide an automated means for on-line high-precision isotope ratios for bulk analysis of solid and nonvolatile liquid samples. Samples are placed in a capsule, typically silver or tin, and loaded into a carousel for automated analysis. The sample is dropped into a heated reactor that contains an oxidant, such as copper oxide for C or S analysis, where combustion takes place in an He atmosphere with an excess of oxygen. Combustion products are transported by flowing He through a reduction furnace for removal of excess oxygen and conversion of nitrous oxides into N_2 . A drying tube is used to remove any excess water in the system. The gas-phase products are separated by GC, and detected nondestructively by thermal conductivity before introduction to the IRMS. Recent work has demonstrated the analysis of S isotopes (Giesemann et al., 1994), where samples must first be converted to BaSO_4 prior to loading into the elemental analyzer. For all elements, ini-

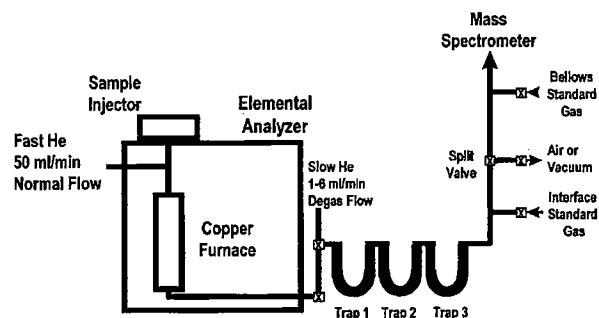


FIGURE 2. Diagram of the *Cryoflow* system for cryofocusing of analyte gases derived from an elemental analyzer (redrawn from [Fry et al., 1996]). Furnace temperature, He carrier flows, and trap conditions are adjusted for optimal analysis of CO_2 , N_2 , or SO_2 . For instance, for CO_2 analysis the furnace is held at 600°C with a slow He flow rate of 2 mL/min , trap 1 is at -86°C to remove water, and trap 2 is either immersed in liquid nitrogen to concentrate CO_2 or $+22^\circ\text{C}$ to release CO_2 . Trap 3 is necessary for SO_2 only.

tial sample sizes in the mg range are most easily analyzed with these instruments.

Oxygen and hydrogen are the two most recent elements for which elemental analyzer data for bulk compounds have been presented. Oxygen-containing samples are converted on-line to CO by pyrolytic reaction with carbon (the "Unterzaucher reaction") as first shown by Brand et al. (Brand, Tegtmeier, & Hikert, 1994), using a GC-based system; several other reports using this principle subsequently appeared, showing elemental analyzer (Koziet, 1997; Werner et al., 1996) or direct injection analysis (Begley & Scrimgeour, 1997). The very recent report of Farquhar et al. (Farquhar, Henry, & Styles, 1997) demonstrates the automated on-line conversion of the oxygen in water or nitrogen-containing plant dry matter to CO, using a pyrolysis-based reaction on nickelized carbon at about 1100°C . CO is separated from N_2 , using a GC with a molecular sieve column; precisions of $\text{SD}(\delta^{18}\text{O}) = 0.2\text{‰}$ are obtained. Begley and Scrimgeour (1997) have shown the analysis of oxygen and hydrogen (δD) on a single sample by measuring the isotope ratio of H_2 gas produced in the pyrolytic reactor with a high mass dispersion IRMS, with an abundance sensitivity that capable of fully resolving analyte HD from excess ^4He carrier. Precisions for water, urine, and volatile organic compounds are about $\text{SD}(\delta\text{D}) = 2\text{‰}$ and $\text{SD}(\delta^{18}\text{O}) = 0.3\text{‰}$.

Low sample size analysis has been an important goal in recent instrument work. Fry et al. (Fry et al., 1996) described an elemental analyzer-IRMS system for measuring C, N, and S isotopic compositions of gas samples in the 10–1000 nmol range. In this CF interface shown in Fig. 2, which the authors refer to as *Cryoflow*, samples are injected or dropped into a quartz tube that is partially filled with reduced copper and that is held at a temperature

of 600–1000°C, with a continuous flow of He. For samples requiring combustion, oxygen was added directly from a tank, or by mixing the sample with V₂O₅, or a combination of both. After reaction in the combustion furnace, gases are transported by normal high flow-rates of He through a series of traps for purification and collection of the target analyte. Once the analyte gas is trapped, the flow-rates are lowered and the trap is heated to permit flow of the analyte into the IRMS. In this interface, all separation is conducted via cryogenic traps; no column is used, as is the case with the standard elemental analyzer interface. In this sense, the system is a hybrid between a CF and batch system, but clearly demonstrates that the CF principle works well with cryotrapped analyte. Sequential analysis of all three elements is possible for a single sample by holding analyte gases in the traps while the IRMS tuning is adjusted to optimize for each element. Straightforward modifications to the IRMS should make possible the automated analysis for all three elements. Precisions of $SD(\delta) < 0.3\text{‰}$ for samples > 50 nmol C, N, or S could be routinely obtained.

B. Trace Gases

Interest in stable isotope measurements of trace gas species, particularly CH₄, N₂O, and CO₂, continues to increase because of the utility of isotopic information for interpreting concerns such as climate change, global warming, O₃ production, and OH depletion (Conny & Currie, 1996; Morse et al., 1996; Ciais et al., 1995). CH₄ carbon isotope ratios in natural systems are indicative of mechanisms and pathways of CH₄ cycling (Sansone, Popp, & Rust, 1997). It is frequently desirable to determine high-precision isotope ratios of environmental gases present at very low ambient concentrations in either gas or dissolved form. An example is atmospheric methane analysis, which often requires 50 L of sample to yield sufficient methane for a precise isotopic analysis (Lowe et al., 1991). Considerable effort has been directed to optimize the detection limits for analysis of environmental trace gases, in the atmosphere, and dissolved in water. To address this problem, an automated cryogenic system, referred to as “PreCon,” was presented recently for analysis of N₂O and CH₄ (Brand, 1995). A 100 mL bulb of sample gas is mounted atop a unit equipped with one manual and two automated liquid N₂ traps that serve to concentrate or purify one of the two gases prior to a final purification by GC, before introduction to the IRMS. CH₄ originally at 1700 ppb in laboratory air was analyzed at a precision of $SD(\delta^{13}\text{C}) < 0.2\text{‰}$, whereas N₂O analysis produced about 0.2‰ for nitrogen and oxygen ratios.

In a very recent report, Sansone and coworkers demonstrate the operation of cryogenic trapping systems that are capable of the automated analysis of 200 pmol CH₄ dissolved in either water (1–50 nmol CH₄) or gas samples

[45–250 ppm CH₄ (v/v)] (Sansone, Popp, & Rust, 1997), as shown in Fig. 3. Water samples are automatically loaded into a sparging column, where dissolved CH₄ is removed by flowing He. Sequential drying and ascarite traps remove water and CO₂, respectively, before the CH₄ is cryotrapped prior to cryofocusing for GC-combustion-IRMS analysis (to be discussed below). Precisions were $SD(\delta^{13}\text{C}) = 0.8\text{‰}$ for replicates of 1.5 or 25 nM CH₄ in seawaters. These authors also describe preparation systems for low-level gas analysis, where trace quantities of CH₄ are purified, preconcentrated, and separated from O₂/N₂ prior to cryofocusing on a GCC-IRMS. Precisions of $SD(\delta^{13}\text{C}) = 0.3\text{‰}$ for a laboratory gas standard of about 100 ppm CH₄ in He are obtained.

C. Breath Tests

The isotopic analysis of breath CO₂ is increasingly important in biomedical applications because of numerous putative diagnostic tests that rely on the measurement of isotopic labeling of breath CO₂ after oral ingestion or injection of a test substance (Halliday & Rennie, 1982). Typically, a substrate is chosen so that the labeled substance is metabolized to CO₂ at a rate that is limited by the process to be measured. The labeled CO₂ is diluted many-fold by a much larger volume of body CO₂ before being expired, leading to very small enrichments over baseline and the attendant requirement for high-precision analysis. The ¹³C breath test is a nonradioactive alternative to ¹⁴C breath tests, and has been validated for use in clinical diagnosis and medical research for the detection of a number of conditions, including pancreatic functional impairment (Watkins et al., 1977), gastric emptying (Ghoos, Rutgeerts, & van Trappen, 1985), and gastric infection by *Helicobacter pylori* (Marshall & Verdun, 1990).

CO₂ must be purified from an excess of water vapor and air gases prior to analysis. Automated systems based on cryogenic purification have been used for years (Schoeller & Klein, 1979), but are slow and cannot be used for samples from subjects whose breath is contaminated with appreciable concentrations of gases that co-condense with CO₂ or that yield isobaric interferences, such as ethanol or ketones. CF analysis of breath was first conducted by manual injection of sample into the carrier flow of an elemental analyzer interfaced to an IRMS (Preston & McMillan, 1988). This system avoided cryogenic purification by using a GC column to purify CO₂. A fully automated system was described soon afterward that samples breath gas from vacutainers along with GC purification (Prosser et al., 1991). Commercial dedicated systems are now available for breath gas analysis from four manufacturers based on very similar principles.

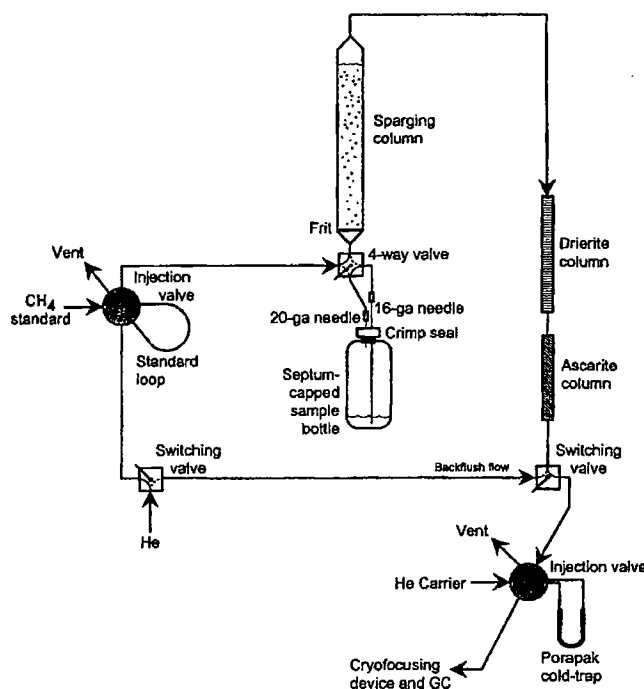


FIGURE 3. Diagram of the automated sparging system from Sansone et al. (Sansone, Popp, & Fry, 1997). Sample water is loaded into a sparging chamber and dissolved gases are removed, purified through drying and ascarite traps, and cryogenically concentrated for analysis. The IRMS (not shown) is located after the GC (reprinted with permission from *Anal. Chem.* 1997, 69, 40–44, Fig. 1, copyright American Chemical Society, 1997).

IV. COMPOUND-SPECIFIC ISOTOPE ANALYSIS (CSIA)

A. Gas Chromatography (GC) and Carbon

The first high-precision GC-combustion-IRMS (GCC-IRMS) based on a multicollector IRMS drew from the early work on GC-combustion (Matthews & Hayes, 1978) to demonstrate that isotopic analysis with a dual collector instrument produced isotope ratios an order of magnitude more precise than demonstrated for an optimized single collector instrument. Barrie et al. (Barrie, Bricout, & Koziat, 1984) used a combustion furnace filled with Co_3O_4 coupled to a cryogenic water trap to prepare CO_2 from a mixture of natural flavoring compounds. Precisions were about $\text{SD}(\delta^{13}\text{C}) = 1\text{‰}$, with a small negative bias of about 1‰ observed for the calibration with CO_2 gas; that bias was nearly eliminated when calibration was conducted against an analyte peak used as an internal standard.

Many acronyms are in use to describe essentially the same fundamental instrument approach. GC-IRMS and GC-C-MS were used for the earliest commercial instruments by separate manufacturers. The term “mass fragmentography” used by Sano and coworkers was replaced by the earliest acronym to be widely adopted, “isotope

ratio monitoring GC/MS” (irmGC/MS) introduced by Matthews and Hayes (1978). The latest may be Compound-Specific Isotope Analysis (CSIA), introduced recently by Schoell (Brand, Tegtmeier, & Hilkert, 1994; Schoell & Hayes, 1994). None of these abbreviations includes the distinguishing characteristic of the technique as now practiced, high-precision, and most could be used to describe other analytical techniques. Because the choice is rather arbitrary and they are not linked to commercial instruments, we use *GCC-IRMS* or *LCC-IRMS*, and high-precision *CSIA* (or *PSIA* below) when a more general term is desirable.

Fundamentals of the modern GCC-IRMS instrument for the analysis of carbon isotopes differ very little from the principles in Matthews and Hayes (1978) and in Barrie et al. (Barrie, Bricout, & Koziat, 1984). Commercial instruments have been available since 1990 with metal oxide combustion furnaces and either Nafion or cryogenic water traps. A schematic of an instrument is shown in Fig. 4 (Goodman, 1997). The GC is an standard capillary system with any of the usual injectors, split/splitless or on-column. The GC effluent is directed to a combustion reactor, which is normally a ceramic or quartz tube with capillary connections at the entrance and exit. The reactor is usually loaded with a metal oxide derived from oxidizing metal wire,

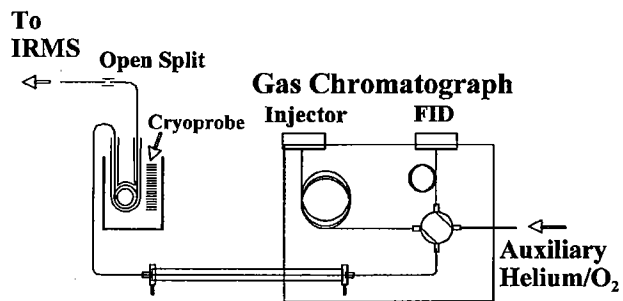
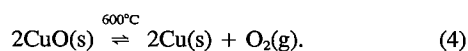


FIGURE 4. Diagram of a GCC-IRMS instrument, after Goodman (1998). A conventional GC is interfaced to a switchable valve that directs the column flow to waste while the solvent peaks elute, or to the combustion furnace when analyte elutes. In the waste mode, He with a small concentration of O_2 for reagent recharge flows through the furnace. The combustion reactor converts organics to CO_2 and H_2O , using CuO reagent and Pt as a catalyst. The capillary is bathed in cryogen to trap water, then is directed to an open split for admission of purified CO_2 to the IRMS.

such as Cu, along with Pt catalyst intended to promote completion of the oxidation reaction. At $600^\circ C$, CuO is a source of O_2 via



As is clear from this equation, CuO is a secondary reagent in the reaction and must be replenished periodically. This replenishing (or "recharging") can be accomplished automatically by including a small amount of O_2 in the stream of carrier gas flowing through the furnace in-between samples (Merritt et al., 1995). It is necessary to divert solvent peaks away from the furnace, because solvent would otherwise prematurely deplete this reagent. Most systems accomplish this diversion by a backflush switching of flow pressure under computer control so that He is forced back through the furnace toward the capillary column (Goodman & Brenna, 1992). Because optimal flow-balancing can be time-consuming, an automated rotary valve can be substituted in place of the backflush. The valve switches solvent flow to waste (or a flame ionization detector) while clean He is flowing through the furnace, or connects the capillary with the furnace once the solvent peak has eluted, as shown in the Fig. 4.

CO_2 and H_2O representative of the combusted organic analyte emerge from the furnace. Water must be removed prior to admission to the ion source because it protonates CO_2 to produce HCO_2^+ , which interferes with analysis at m/z 45. Either a NafionTM tube or a cryogenic trap is employed to remove the water of combustion. A Nafion-type trap consists of a length of the fluorinated polymer tube attached to a capillary at its entrance and exit. The analyte stream passes into the tube, where water passes through

the walls and is swept away with counterflowing He, while CO_2 is quantitatively retained. This trap offers the advantage that it operates continuously at room temperature without attention. Its principle disadvantage is that it introduces two connections, which offer the possibility of leaks, as well as two diameter changes, which compromise chromatographic resolution.

The cryogenic trap shown in Fig. 3 consists simply of a length of capillary or tubing bathed in an appropriate cryogen, which retains water but passes CO_2 . This approach has the disadvantage that the trap must be warmed periodically to remove trapped water. However, as shown in Fig. 3, it offers the possibility of using a continuous capillary from the end of the GC column through the combustion furnace and water trap and directly to the open split leading to the IRMS. In this design, the oxidant wire is threaded into the capillary to an appropriate point, and the capillary is anchored at each end of a ceramic tube. One end of the capillary is attached to the rotary valve in the instrument, and the end emerging from the furnace passes through a cryogen bath and to the open split. When the furnace is first operated, the protective polyimide coating outside the capillary burns away to leave a bare fused silica capillary that is mechanically stabilized at the furnace temperature by the ceramic tube. This system is resilient in use over many months as long as the furnace temperature is maintained, and offers the advantage of best-case chromatography and leak-free operation as long as capillary continuity is maintained. The open split referred to above is required to feed analyte gas at a stable flow rate to the IRMS; splits of this sort are common in MS applications.

High column bleed is a particular problem in GCC-IRMS, because the combustion furnace converts all organic compounds to CO_2 before analysis. Unlike the organic MS, no additional separation based on mass analysis is possible. Hence, high bleed rates translate into high backgrounds and diminished dynamic range.

Carbon isotope data from a single GC peak are shown in Fig. 5. Three traces, representing masses 44, 45, and 46, are recorded as the analyte CO_2 passes through the ion source. The peaks are intentionally of comparable magnitude because of the mismatch of amplifier gains that was mentioned previously. For CO_2 , the relative gains in the Finnigan MAT 252 are 3 to 300 to 1000 for m/z 44, 45, and 46 channels, respectively. The ratio of m/z 45 to 44 is plotted above the chromatograph to illustrate the commonly observed phenomenon that the 45 peak is observed to elute prior to the 44 peak, yielding a "positive-negative going" ratio trace. This reversal is thought to be due to the differences in intermolecular interactions between the isotopically substituted peaks and all ^{12}C peaks. These traces are used to generate integrated areas by applying either the summation or curve-fitting algo-

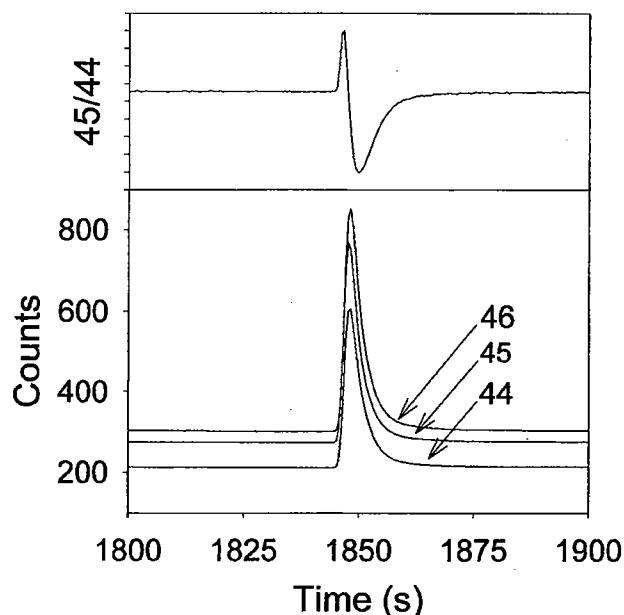


FIGURE 5. Traces of the m/z 44, 45, and 46 mass channels as a GC peak are analyzed in the IRMS. The signals are of comparable size, because the amplifications are set to match natural abundance. Peaks are integrated and areas are used to calculate isotope ratios.

gorithms, as discussed above. Isotopic calibration can be accomplished if one of the peaks in the chromatograms is an internal standard (Caimi, Houghton, & Brenna, 1994). More commonly and conveniently, a pulse of calibrated CO_2 from a gas bottle is admitted to the ion source through a separate inlet during a period where no peaks elute from the GC. Precisions of $\text{SD}(\delta^{13}\text{C}) < 0.3\text{‰}$ are routine for well-resolved, strong peaks that are integrated and calibrated in this way. For carbon, the minimum sample size required to yield a precision of this level is about one nmole carbon (Merritt et al., 1995; Goodman & Brenna, 1992; Merritt & Hayes, 1994).

One of the analytical limitations of GC-combustion is the dynamic range over which enrichments can be accurately and precisely measured. In our hands, carbon isotope ratios much above $\delta^{13}\text{C} = 1000\text{‰}$ will compromise precision and accuracy, partly due to the large difference in isotope ratio between them and natural abundance standards. This problem can be remedied in part by the use of enriched standards; however, this measure does not overcome the mismatch in amplifications among the channels, which, as noted previously, are set to make natural abundance signals of nearly equal magnitude. It is very difficult to overcome this problem, because the amplifiers must be set simultaneously to the ratio anticipated for the enriched samples and for the baseline samples, which inevitably are analyzed in the same chromatogram.

An alternative solution to this problem has been pre-

sented, where the GC is simultaneously coupled to a combustion-IRMS system and an ion trap mass spectrometer (Meier-Augenstein et al., 1994; 1995; Meier-Augenstein, 1995). The flow of separated analytes emerging from the capillary column is sent through a splitter, which sends about 10–20 parts to the combustion-IRMS and one part to the ion trap. The split ratio is chosen in these proportions, because the ion trap can detect with ease sub-nmoles of most organic compounds, whereas the IRMS requires higher analyte levels to maximize counting statistics. Excellent linearity was achieved throughout the low and high enrichment ranges, indicating that this approach is the means of choice for wide dynamic range determination of carbon isotope ratios.

B. CSIA/CF Data Analysis

A separate discussion of data analysis for CF data is warranted and is best considered for GCC-IRMS of carbon, for which most of the work has been done and is suitably representative of all but hydrogen analysis. One of the commercial data analysis algorithms in routine use has been published in some detail (Ricci et al., 1994), and is based on the most common algorithm used for chromatographic peak analysis, known as the “summation” method. The start of a peak is identified in the highest abundance mass channel trace (m/z 44 for CO_2) by comparing the slope from a rolling five-point regression to a user-defined threshold, and the end of a peak is determined as the first point after the apex that drops below some fixed percentage of the apex maximum. The most abundant peak is used, because it will have the highest signal-to-noise (S/N) ratio. Peaktops are defined in the other mass channels. Because of the slightly differing chromatographic elution times of isotopomers, the mass scales of the other channels are adjusted so that apices of all mass channels correspond in a procedure known as “time-shifting,” in order to identify the peak starts and stops. The background can be identified and subtracted, and the excess peak area summed to yield the abundances. These peak areas are ready for calibration. This procedure works well near natural abundance, but is less effective at very high enrichments, where the area of the minor isotope peak, for instance m/z 45, is of disproportionate size and is not subsumed by the major isotope. Tails of the m/z 45 peak extend well beyond the limits of the m/z 44 peak, and are not integrated. The solution to this problem is to use the m/z 45 peak for detection and to time-shift the m/z 44 channel. This process, in our experience, compromises little in terms of effective peak detection but significantly improves the results.

There are a few published studies on various issues and data processing. We investigated the effects of incomplete chromatographic resolution on the calculation of iso-

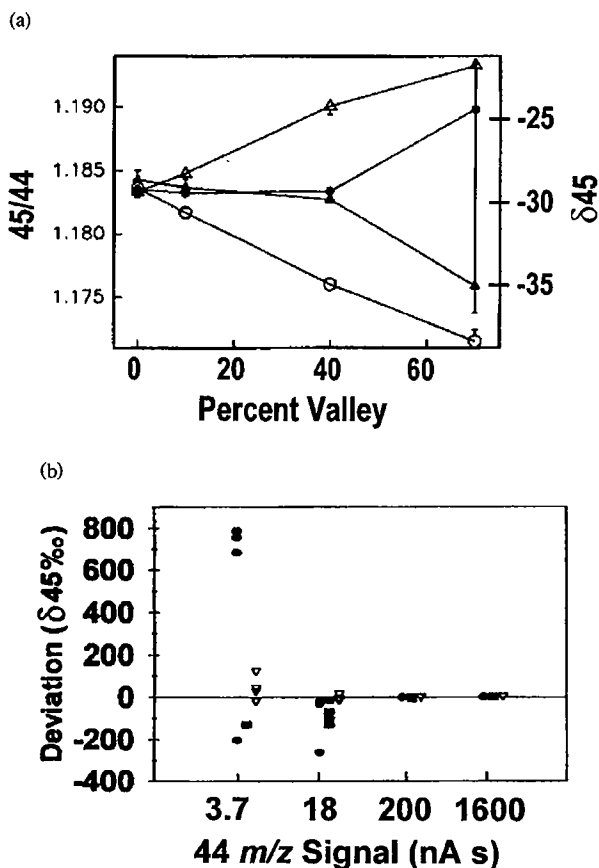


FIGURE 6. (a) Results of a study of the effect of peak overlap on isotope ratios calculated from areas derived by the summation method (open symbols) or by curve-fitting (closed symbols) (Goodman & Brenna, 1994a). Two compounds (methyl tridecanoate and butylated hydroxytoluene) of nearly equal $\delta^{13}\text{C}$ are analyzed individually and under chromatographic conditions producing graded overlap. Isotope ratios determined by summation start to diverge from best values at 10% valley even though precision is unaffected. Curve-fitting using the exponentially modified gaussian function retains precision and improves accuracy. (Reprinted with permission from *Anal. Chem.* 1994, 66, 1294–1301, Fig. 5, top left panel, copyright American Chemical Society, 1994.) (b) Results of a study of curve-fitting on well-resolved, low signal-to-noise peaks (Goodman & Brenna, 1994b). Curve-fitting (open symbols) produces considerably better accuracy and precision than conventional summation (closed symbols). (Reprinted from the *Journal of Chromatography*, Vol. A 689, Fig. 3, pp. 63–68, 1995, with kind permission of Elsevier Science–NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.)

tope ratios by the summation method and by curve-fitting, using several functions designed for chromatographic applications (Goodman & Brenna, 1994a). This procedure consists of nonlinear least-squares fitting, using the Marquardt–Levenberg algorithm to interrogate parameter space. Goodness-of-fit parameters are calculated for each set of fitted coefficients and the best values are determined by iteration. The final, fitted-area parameter is used in

further ratio calculations. Figure 6(a) shows the results of increasing peak overlap on calculated isotope ratios for two compounds, methyl tridecanoate (Me13:0) and butylated hydroxytoluene (BHT) that were separated using a capillary column, combusted on-line, and admitted to the IRMS. The two compounds had carbon isotope ratios that matched to about 0.5‰ of one another. For an equal abundance mixture and at an overlap as small as 10% valley, the isotope ratios are significantly different, with the earlier-eluting compound apparently depleted and the latter-eluting apparently enriched by about 1–2‰. At 70% overlap, the isotope ratios have diverged so that they are 7–10‰ from the correct value. The remarkable observation is that the precisions are excellent for all but the 70% valley case, indicating that even small amounts of overlap have a dramatic effect on isotope ratios calculated from areas using the summation method. The degradation in accuracy is *not* apparent from any degradation in the precisions. The effect is even more pronounced for the smaller of a pair of mismatched peaks. Figure 6(a) also shows that curve-fitting with a common chromatographic function, the exponentially modified gaussian, recovers the accurate isotope ratio without sacrificing precision, up to a 40% overlap.

Further work on curve-fitting (Goodman & Brenna, 1994b) showed that precision could be preserved at very low signal levels with curve-fitting for well-resolved peaks, as illustrated in Fig. 6(b). Curve-fitting's strength is well-known to be superior for low S/N data, and our results confirmed this observation for CF isotope ratio analysis—as shown. Thus far, curve-fitting has not been incorporated into any commercial IRMS data reduction software.

Others have shown that the time-shift procedure is critical to preserve accuracy when chromatographic parameters are altered (Meier–Augenstein, Watt, & Langhans, 1996). For example, the alteration of temperature-ramping parameters, which produce an alteration in background slope due to column bleed, caused changes in calculated isotope ratios. The most straightforward method to deal with this problem is to employ an isotopically calibrated standard that elutes at precisely the same time as the analyte. However, this procedure is much less convenient than the simple inclusion of a pulse of isotopically calibrated gas at an uncluttered section of the chromatogram.

C. GC and Nitrogen

In 1994, descriptions of two systems were published that demonstrated the high-precision CSIA of N (Preston & Slater, 1994; Merritt & Hayes, 1994) on multi-collector instruments, and that applied to amino acid analysis. The two most important challenges to N analysis are the very

low concentration of N in organic compounds compared to C, and the necessity to prepare N_2 , which requires reduction chemistry, as opposed to C, which requires oxidation. The system of Preston and Slater directs the output of the analytical GC to a conventional combustion furnace, which converts volatile amino acid derivatives into CO_2 , CO , H_2O , and N_2 with, presumably, nitrogen oxides as well. A cryogenic trap removes water, CO_2 , and higher oxides of N, and a second GC separates N_2 from CO . That overlap would be a serious isobaric interference at m/z 28 if CO were permitted to enter the IRMS. For seven nmol of phenylalanine, these authors report a precision of $SD(\delta^{15}N) = 5\%$ by using an internal standard, and they report that this precision is limited by the precision of their IRMS.

Merritt and Hayes reported a similar system with the addition of a reduction reactor loaded with Cu wire to reduce N-oxides to N_2 and to remove O_2 , and with the use of a higher precision IRMS. Their system also included a combustion furnace and cryogenic trap for water removal, and it produced a precision of $SD(\delta^{15}N) = 0.2\%$ for a sample size of two nmol of an amino acid, with an accuracy better than 0.05% . This system's performance was superior at the expense of added complexity.

D. General CF Analysis of Hydrogen

The methodology for CF analysis of C and N isotopes involves advanced on-line sample chemistry with microreactors and separation traps, and can be applied to aspects of hydrogen isotopic analysis. However, obstacles to CF-IRMS of hydrogen isotopes, such as memory effects due to water, make H a more formidable goal. For instance, experience with a dual IRMS that was introduced in the mid-1980s and designed to analyze water samples directly injected into an inlet suffered severe memory effects and required 6–20 injections between sample changes to flush the system of any remaining previous sample (Wong, Cabrera, & Klein, 1984). This system did not use an He carrier, and, therefore, was not a CF system in the modern sense.

In CF methodology, the inert carrier gas, usually pure He, is used to sweep the analytes of interest into the mass spectrometer. He presents another challenge for IRMS H analysis because it interferes with an HD measurement in two ways. First, standard laboratory grade He gas contains ~ 1 ppm 3He isotope that adds to HD measurement, but can be corrected for by background subtraction. Second and much more important, the abundance sensitivity of conventional IRMS is not great enough to resolve HD from the tail of a much greater $^4He^+$ beam. The $^4He^+$ tail often results in a saturated m/z 3 detector. Other obvious inert gas choices that might be used as a carrier, such as Ne, Ar, and Xe, have numerous disadvantages including

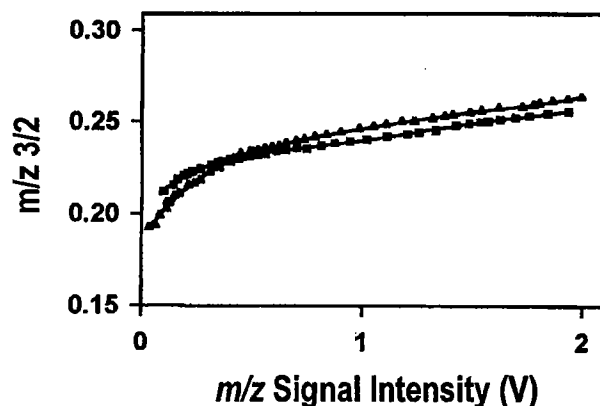


FIGURE 7. Effect of coadmitted N_2 on hydrogen isotope ratios. The presence of N_2 co-admitted to the ion source with H_2 (▲) measurably alters the ion current ratios determined for HD/H_2 , compared to pure H_2 (●), in part because of isotope-dependent reactions in the ion source. Hydrogen, including contaminants from, for instance, combustion/reduction of N-containing compounds, will be subject to this bias and may not be effectively calibrated using pure gas.

(1) compromised chromatographic resolution due to their high mass, (2) very high densities and low ionization potentials compared to He, leading to high-intensity ion beams that rapidly damage ion optical components and insulators that lead to electronic problems (Tobias et al., 1995), (3) for Ne and Xe, very high cost, and (4) sensitivity of isotope ratio to co-admitted contaminants. The latter effect is demonstrated in Fig. 7, where a co-admitted contaminant, N_2 , produces a statistically different D/H than for pure H_2 . This difference can be an important consideration in organic analysis using chromatography, where heteroatoms such as N or S will be converted to stable species that will be admitted to the ion source along with the analyte gas unless a purification step is included.

In 1995, Prosser and Scrimgeour (Prosser & Scrimgeour, 1995) introduced a novel mass spectrometer with an extra collector spur, using a large radius that achieves a high dispersion of m/z 3 from m/z 4. The diagram in Fig. 8 shows the simultaneous detection of the m/z 2 and 3 beams, whereas the m/z 4 beam (not shown) is not sufficiently deflected in the magnetic field and collides with the flight tube walls. This configuration yields remarkably high abundance sensitivity, and prevents any measurable interference from 4He without resorting to elaborate high-vacuum pumping systems common in high abundance sensitivity TIMS instruments.

Another approach developed at the same time by Tobias et al. (1995) is the Pd filter system (PFS), in which carrier is completely prevented from entering the IRMS ion source. A Pd foil membrane held at elevated temperatures is used to selectively admit only hydrogen and deuterium into the MS while diverting the carrier gas to waste.

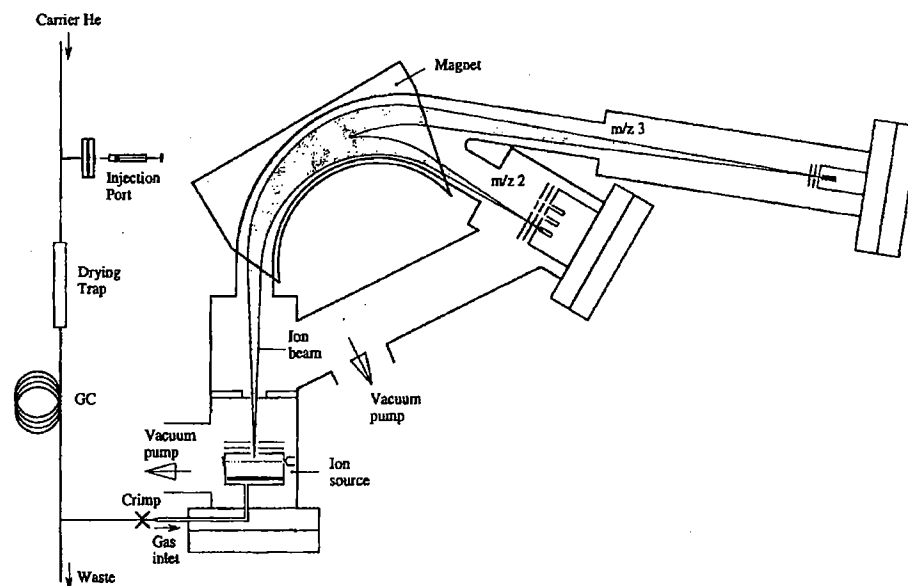


FIGURE 8. Diagram of a high-abundance sensitivity IRMS tailored to the CF analysis of hydrogen in He carrier gas (Prosser & Scrimgeour, 1995). The tail of the very large ^4He normally interferes with HD at m/z 3 is eliminated using very high dispersion. H_2 at m/z 2 and HD are detected simultaneously while ^4He at m/z 4 collides with the vacuum chamber walls. (Reprinted with permission from *Anal. Chem.* 1995, 67, 1992–1997, Fig. 1, copyright American Chemical Society, 1995.)

This membrane can be made of pure Pd metal, but more often is a Pd alloy. A vanadium alloy, with a thin plate of Pd on both surfaces, can also be used to facilitate greater permeability and lower dependence of isotope permeability on temperature (Tobias & Brenna, 1996). These two developments facilitate the CF-IRMS of H isotopes and permit the development of on-line chemical techniques for specific sample analyses.

E. Water Analysis

Prosser and Scrimgeour demonstrated a CF-IRMS method for water analysis with their high dispersion instrument. They applied the method of Horita (Horita et al., 1989), wherein water and hydrogen gas are equilibrated with a Pt catalyst (Prosser & Scrimgeour, 1995), and report precisions of $\text{SD}(\delta\text{D}) < 4\text{‰}$ for the resulting hydrogen. Very recent, Begley and Scrimgeour reported a pyrolysis system for CF analysis of either water or volatile organics (Begley & Scrimgeour, 1997). Their system used carbon-coated nickel to convert water or volatile organics to H_2 and CO for the analysis of D/H or $^{18}\text{O}/^{16}\text{O}$, respectively. The IRMS was a high-dispersion instrument with sufficient abundance sensitivity to fully separate the tail of ^4He from HD. This system produces $\text{SD}(\delta\text{D}) = 2\text{‰}$ and $\text{SD}(\delta^{18}\text{O}) < 0.3\text{‰}$ for water or organic samples. A nickel plug inserted in the injection port prevents salts and nonvolatile organic matter from entering the system, and permits the direct

analysis of urine with a precision equivalent to that observed for water. Some memory effects of $<2\text{‰}$ are observed for both isotopes. This type of system has an inherent potential advantage over a PFS, because more than one isotope can be analyzed in a single analysis.

The direct analysis of pure hydrogen gas, using the PFS with a pure Pd membrane, results in a precision of $\text{SD}(\delta\text{D}) < 6\text{‰}$. These data result from sample sizes that were matched to avoid nonlinearities associated with hydrogen isotopic analysis—that topic will be addressed in detail below. An on-line chemical reduction scheme developed by Tobias et al. can be used for direct water analysis for its D/H after conversion to H_2 (Tobias et al., 1995). Water samples (100 nL) are introduced into an He carrier stream via a GC injector, and are subsequently reduced to hydrogen gas by the use of a micro-reactor that is filled with Ni metal and is held at $>850^\circ\text{C}$. The use of a Nafion water trap eliminates problems associated with any residual unreduced water vapor, and it does not affect isotopic measurement. There is no evidence of memory effects over a $\delta\text{D} = 5000\text{‰}$ range in isotope ratio. This on-line chemical configuration is compatible with the high-dispersion instrument (Begley & Scrimgeour, 1996) and makes it convenient for doubly labeled water samples, because it can be used to analyze the isotopes of O and H from water by using the same instrumental configuration. Nickelized carbon can be used to generate CO from H_2O to allow for the analysis of $^{18}\text{O}/^{16}\text{O}$ from CO.

F. CF Analysis of Hydrogen: Correction Procedures

One of the critical issues facing CF H analysis is the presence of two nonlinear effects that result in ion current ratios that depend on the chemical composition in the ion source. One of these factors is the pressure-dependence mentioned previously and common to all IRMS elements. That dependence is effectively eliminated by the carrier gas background pressure. In chromatography experiments, CF analyte levels are difficult to match between different analyte components in a sample mixture and usually vary over a large range. This variation is an important consideration for hydrogen analysis as a result of the false contributions to the m/z 3 signal due to effects in the tight IRMS ion source that change with varying analyte quantity. The situation contrasts with dual inlet analysis, where sample and standard pressures are always equalized and such problems are easily addressed. Ion current ratios depend on the total gas pressure in the ion source due to pressure-dependent mass selection (Kirshenbaum, 1951). The selectivity toward the higher mass ions is observed because of the interaction of the fields from the collimating magnets on the acceleration of ions. In effect, the ion source acts like a small mass spectrometer. Space charge, which increases with the amount of analyte gas in the ion source chamber, also contributes to mass discrimination (Scrimgeour et al., 1993). For hydrogen, the second and much greater effect is the production of H_3^+ in an ion-molecule reaction that depends on hydrogen-hydrogen collision frequencies:



The H_3^+ formed in this reaction is proportional to the square of the amount of H_2 in the ion source, and is not resolved from HD in commercial IRMS instruments.

Empirical correction procedures for these effects have been proposed for over a 2-fold signal intensity range by Prosser and Scrimgeour (1995), and up to a 22-fold range by Tobias et al. (Tobias & Brenna, 1996). These effects depend on specific instrument tuning parameters. Any nonlinearities that result from, for example, unmatched m/z 2 and 3 detector time constants, can complicate correction procedures by introducing another second-order dependence of measured m/z 3/2 ion current ratios on m/z 2 peak area. Matching of detector responses results in a linear dependence and makes correction procedures more straightforward. In these procedures, the characterization and correction of ion source effects are conducted after hydrogen peak detection and isotope ratio calculation. Such procedures are referred to as *peakwise* to reflect their application after peak detection. These routines require the analysis of analyte over a wide signal range to characterize

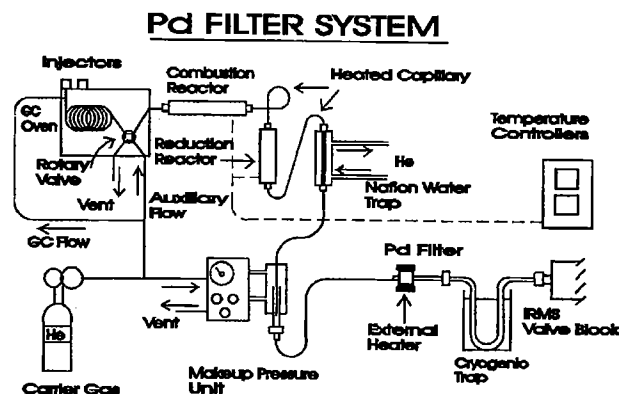


FIGURE 9. Diagram of the Pd filter system (PFS) for CSIA of H using a GC. The GC effluent is sequentially combusted and reduced, and the flow is stepped up using a modified GC injector, and directed onto a Pd-V-Pd sandwich foil held at 600°C. Hydrogen and its isotopes diffuse through the foil at an efficiency of about 20%, while the carrier stream and any contaminants are vented to waste. (Reprinted with permission copyright American Chemical Society, 1996.)

and correct the nonlinearities. A *pointwise* correction, where characterization and correction of ion source effects are conducted before hydrogen peak detection and isotope ratio calculation, should not require correction data over a wide signal range. In our hands, a pointwise procedure was not effective, seemingly due to unknown effects from continuously varying signals (Tobias, 1997). Peakwise correction procedures prior to isotopic calibration are in current use for CF H analyses (Begley & Scrimgeour, 1997).

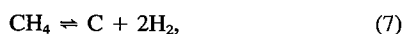
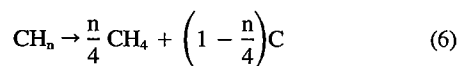
G. CSIA for Hydrogen

Tobias and Brenna (1996) first demonstrated CSIA for H using the PFS coupled to GC, with an apparatus shown in Fig. 9. An on-line combustion microreactor, filled with CuO held at 850°C, and a reduction reactor filled with Ni metal held at 950°C, are used to convert separated organics to hydrogen gas. Evaluation of the system, using benzene as an internal standard in a mixture of ethyl benzene and cyclohexanone in hexane, results in precisions of $SD(\delta D) < 5\%$ with organic analytes at quantities of < 3 ng (< 300 pg H). Similar results are demonstrated in a procedure developed for hydrogen isotopic analysis of components in natural gas mixtures using the PFS.

Long capillary columns are often required when analyzing complex mixtures of organics. We found that long capillaries lead to lower flow-rates and carrier pressures at the Pd filter, resulting in a decreased hydrogen sensitivity. A make-up pressure unit was inserted between the on-line reactors and the Pd filter to increase the pressure with which the carrier and analyte impinge upon the Pd surface to facilitate an increase in hydrogen transfer.

There are at least two serious problems with the on-line chemical conversion of organics to hydrogen. First, chemical reductants used to date have very limited lifetimes; in our hands, 8 h or so. This limitation is exacerbated by the emission of O₂ from the obligatory combustion reactor placed in-line prior to the reduction reactor. Besides being a nuisance, frequent recharges of the reduction reactor usually require system recalibration; that step costs additional time. Second, chemical reduction by the addition of atomic oxygen to a solid metal implies a reaction that depletes the reagent's active surface. This depletion seems to manifest itself in a time-dependent passivation of the reducing capacity, which recovers with a time constant of minutes. Because the reagent eventually must be replaced, this phenomenon is consistent with the solid state diffusion of oxygen into the bulk metal and a restoration of reducing capacity as bulk metal diffuses to the surface.

Whether or not this explanation is correct, there is clearly a need for an alternate means to rapidly convert organic H to H₂ in a robust, on-line manner. Sofer has presented high temperature as a means to generate H₂ from hydrocarbons, and has shown that high temperature produces a precision of SD(δ D) < 3‰ when implemented off-line in sealed reaction tubes (Sofer & Schiefelbein, 1986). The chemical reactions for hydrocarbons are



thus demonstrating that the reaction proceeds via methane. Above about 1000°C, the latter reaction favors the products and the equilibrium constant is about 100 at 1200°C. The main advantage to this strategy is that it can be used as a limitless reduction source, and that it results in a more robust and low maintenance CF-IRMS system for hydrogen. In our recent work on such a system (Tobias, 1997), an empty alumina tube heated to 1150°C (a safe maximum temperature for the existing furnace in the system) replaces the two on-line chemical reactors. Tests to determine whether the 600°C Pd filter plays any catalytic role in reduction showed that nearly all of the measurable hydrogen signal is due to conversion in the hot pyrolysis furnace. For small hydrocarbons in natural gas mixtures, precisions of isotope ratio measurements are shown to average SD(δ D) < 2‰ because of reduction reproducibility. In addition, isotopic measurement stability was excellent over 24 h, and it appears to be indefinite, dependent only on instrument stability. The disadvantages of the technique are that the maximal pyrolysis temperature varies with compound type. That variability may result in a dif-

fering fractionation of hydrogen isotopes between different compounds. The possible role of heteroatoms giving rise to hydrogen-containing products such as H₂O may also induce fractionation. These problems can be remedied by the inconvenient but effective procedure of calibration for each compound of interest. For chromatography, the PFS offers the advantage that co-eluting contaminants, such as N₂, do not enter the vacuum system along with the analyte hydrogen. In addition, the PFS prevents instrument contamination from unreduced organics due to inefficiencies of thermal degradation with on-line pyrolysis. Ion source contamination may be particularly important for pyrolysis-induced H₂ generation from compounds with heteroatoms.

H. Liquid Chromatography-Combustion (LCC)-IRMS

The major limitation of GC-based systems is their requirement for a volatile and thermally stable analyte. Although nonvolatiles are routinely chemically derivatized prior to GC analysis, most derivatizing agents add extraneous C, H, O, or N to the molecule that cannot be distinguished from the analyte elements by the IRMS, and, therefore, must be taken into account in later calculations to yield accurate analyte isotope ratios. Isotopic fractionations existing at reaction centers can substantially complicate the correction procedure by altering the analyte carbon isotope ratio by an unpredictable degree. These considerations are particularly applicable to biomolecules, which, with few exceptions, require derivatization or simply cannot be made volatile.

The most critical feature for an LC interface is a reliable and reproducible solvent removal prior to analyte introduction to the combustion furnace. Of the several popular approaches to LC-MS interfaces, the moving transport and particle beam devices are the only ones that include an effective desolvation stage prior to introduction to the MS. Prior to its applications to MS, moving transports were used to interface LC to flame ionization detectors (FID), and about 200 were built and sold commercially in the 1960s and 1970s, making it the only interface known to remove solvent well enough for a solvent-sensitive detector. Common to FID and IRMS interfacing is the necessity to remove solvent and to combust analyte at atmospheric pressure. Those requirements are more modest than for organic MS interfacing, which requires a series of vacuum chambers to move the belt into/out of the vacuum system and a means to desorb intact analyte molecules once in the vacuum system. In 1991, Moini and Abramson were able to couple the moving transport interface to demonstrate LC/CRIMS on a conventional single collector organic MS (Moini & Abramson, 1991). We, therefore, initially pursued the moving transport approach to test whether it could be used in a high-precision system.

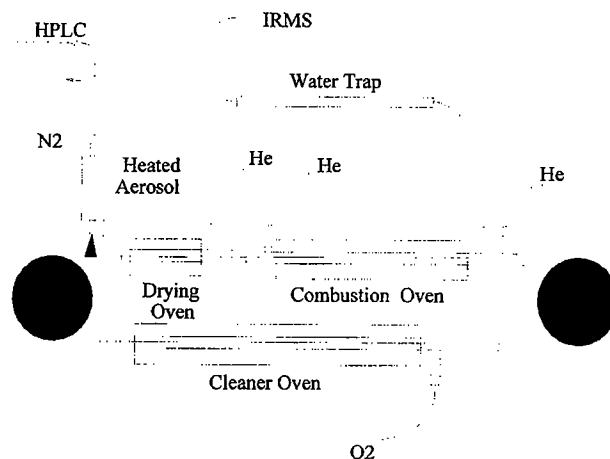


FIGURE 10. Diagram of a moving belt combustion interface for LCC-IRMS (Caimi, 1995). The continuous loop stainless steel belt is cleaned in a furnace and the LC eluent is pneumatically sprayed onto the belt. The belt passes through a drying oven to remove solvent and a combustion furnace to convert analyte to CO_2 and H_2O , where He carrier sweeps the bands of gas to a Nafion water trap, and to the IRMS. No open split is necessary after the water trap, because the furnace is open to atmosphere and serves as a split.

1. Moving Transports

To extend compound-specific carbon isotope analysis to nonvolatile and thermally labile molecules, we introduced an interface that couples liquid sources, including liquid chromatography (LC), with high-precision combustion-IRMS (LCC-IRMS) (Caimi & Brenna, 1993; 1995). In our original transport system, a solution containing the analyte of interest was coated onto a precleaned moving wire, solvent was reproducibly removed in a drying oven, and the analyte was quantitatively combusted in a CuO/Pt -charged furnace maintained at 850°C . CO_2 and water bands that result from analyte combustion were transferred via a sampling capillary to a Nafion-based water trap. Following water removal, the analyte CO_2 was admitted into the IRMS ion source as a series of bands in He carrier gas. Precision and accuracy for this system were comparable to that of GC-based systems for 50-mg samples. Hardware improvements in the original LC interface system resulted in a sensitivity enhancement of about 300-fold (Caimi & Brenna, 1995). However, a primary limitation of wire-based transport systems is poor sensitivity due to the limited capacity of the wire to capture more than 1–3% of the total analyte. Belts are well-known to retain far greater levels of analyte, particularly in pneumatic spray systems, in which the analyte fans out and is not contained within a small volume.

We describe here in some detail the most recent version of the moving transport interface (Caimi & Brenna, 1995). The transport is a continuous-loop, moving belt that captures an estimated 20% of the total analyte supplied. The prototype moving belt interface is shown in Fig. 10. The belt ($0.08\text{ mm} \times 1.5\text{ mm} \times 115\text{ cm}$) first

passes through a cleaner furnace (900°C) that is purged with a parallel flow of O_2 (15 mL min^{-1}), loops around a pulley, and enters an enclosed coating block. In the block, the analyte solution is converted into a heated (150°C) pneumatic spray that is directed at a 45° angle at one surface of the belt, as described previously in detail (Caimi & Brenna, 1995). Any solvent remaining after the coating process evaporates as the belt subsequently passes through a 12-cm drying oven (200°C) purged with He gas (100 mL min^{-1}). Analyte material deposited on the belt is combusted into CO_2 and H_2O as the belt passes through a CuO/Pt -charged furnace maintained at 850°C . The furnace consists of an unrestricted quartz tube ($2\text{ mm i.d.} \times 20\text{ cm}$ long) with two He purge inlets (leading and lagging flows) maintained at a flow rate of approximately 30 mL min^{-1} . A third port is used to sample the products of combustion and to transport them to a 10-cm long Nafion-based water trap. The dried CO_2 is transferred to the IRMS instrument. All moving belt studies reported here were done in flow injection mode without an in-line chromatography column. Accuracy and precision of the isotope ratio measurements were assessed using multiple injections of isotopically calibrated linoleic acid (2.5 mg) standards with graded $^{13}\text{C}/^{12}\text{C}$ ratios.

Peak-shapes obtained with the wire- or belt-based LCC-IRMS interfaces are comparable, and indicate that band-broadening can be minimized. Compared to the best published wire system (Caimi & Brenna, 1995), detectability in the belt system is improved 40-fold. This improvement can be directly attributed to the ability of the belt to capture a larger proportion of the total analyte compared to the wire systems. Injections of as little as 25 ng of

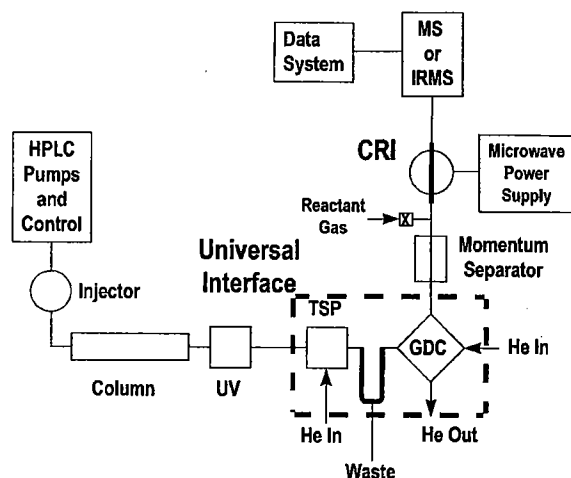


FIGURE 11. Diagram of the LC/CRI/MS interface, redrawn after McLean (McLean et al., 1996). LC eluent flows sequentially through a thermospray pnebulizer (TSP), a solvent trap, a gas diffusion cell (GDC), and a momentum separator to prepare desolvated analyte particles for admission to the chemical reaction interface (CRI) unit. The CRI couples microwave power and reagent gases to convert analyte into small molecules such as CO_2 , which are directed to the IRMS for analysis. The system can also be interfaced to GC.

analyte yield signals clearly discernible from baseline noise, although peaks below the 250-ng level could not be reliably detected or defined by the standard peak identification software. The response of this interface is linear ($r^2 = 0.993$) for analyte loads ranging from 250 ng to 5 mg. Using conventional data reduction, the LCC-IRMS system yields a precision of $\text{SD}(\delta^{13}\text{C}) < 1\text{‰}$ for analyte loads $> 1 \mu\text{g}$. However, as observed previously with both wire systems, with low sample loads, in this case $< 2 \mu\text{g}$, the isotope ratio deviates significantly from the known value that is calibrated against a gas pulse. Biases of this nature have been observed previously in the analysis of low signal levels with GCC-IRMS (Goodman & Brenna, 1994b), but they appear at much lower sample levels. The improvement in GCC-IRMS data with curve-fitting inspired its use for this application, and we have observed similar improvements in LCC-IRMS data quality (Caimi, 1995).

2. LC/Chemical Reaction Interface (CRI)/IRMS

Abramson and coworkers have reported coupling a thermo-spray/particle beam interface with CRIMS to produce an analyte stream from LC (and GC) that is suitable for analysis with a high-precision IRMS (Teffera et al., 1996; Abramson et al., 1996), referred to by the authors as HPLC/CRI/IRMS. Figure 11 is a diagram of the system and is adapted from a non-IRMS publication from the same laboratory (McLean et al., 1996). The eluent from

an LC is directed to a thermospray (TSP) vaporization stage, which volatilizes most of the solvent that condenses on the walls of a desolvation chamber. Analyte particles entrained in a high flow of He are carried through a longitudinal membrane-based gas diffusion cell (GDC). Finally, they pass through a momentum separator, which is the final desolvation stage, and out to a CRIMS interface. The *Universal* interface is the commercial name for the coupled TSP/GDC. Compared to the moving transport, an important advantage of the CRI/IRMS interface is the absence of any mechanical moving parts. Possible concerns are the satisfactory removal of solvent and the absence of nonlinear isotope effects in the plasma chemistry, including reactions that do not proceed to completion.

Replicate injections of $1.2 \mu\text{g}$ of D-ribose into the HPLC introduced about 120 ng into the IRMS to yield precisions of $\text{SD}(\delta^{13}\text{C}) < 1\text{‰}$. There was some small dependence of the isotope ratio on the amount injected between 10 and $20 \mu\text{g}$, a dependence that the authors speculate to be due to a mass interference, possibly $\text{C}_2\text{H}_5\text{O}^+$, arising when CRIMS reactions are not complete. Replicate injections of $2 \mu\text{g}$ chlorophyll-a from two different sources, spinach and algae, produced $\text{SD}(\delta^{13}\text{C}) = 0.5\text{--}0.6\text{‰}$ when calibrated against an internal standard. These two sets of data were found to be significantly different. Solvent removal and isotope effects did not appear to affect the performance in these experiments.

To summarize the state of LC-based CSIA, systems based on two different principles have been demonstrated to yield high-precision results with sample sizes in the one μg range. No commercial versions of these instruments are yet available, although there is some commercial interest in the idea (Brand, 1996). It is probable that considerable work remains to bring LC to the high-precision applications community. It should be noted that tracer applications with more modest precision and accuracy requirements may benefit first from these advances.

TABLE 1. Isotope ratio results from the moving belt interface demonstrate high-precision below 5000-ng load, using either summation or curve-fitting, and systematic bias at lower levels when calibrated against pure CO_2 admitted to the IRMS from a gas tank.

| Load (ng) | Summation | Curve-fit |
|-----------|-------------------|-------------------|
| 5000 | -27.67 ± 0.1 | -27.69 ± 0.10 |
| 2500 | -27.23 ± 0.32 | -26.94 ± 0.08 |
| 1000 | -16.30 ± 0.97 | -16.27 ± 0.86 |
| 750 | -12.67 ± 3.04 | -13.62 ± 2.02 |
| 500 | -13.77 ± 0.49 | -13.63 ± 0.39 |
| 250 | -7.62 ± 1.71 | -7.35 ± 1.1 |

V. POSITION-SPECIFIC ISOTOPE ANALYSIS (PSIA)

Position-specific measurements, that is, measurement of intramolecular isotope ratios, for detection of *natural* variability, have been published but are generally limited only to the most important problems because of the cumbersome chemical degradation and separation steps that are required prior to IRMS analysis. The earliest position-specific research is that of Abelson and Hoering (Abelson & Hoering, 1961), who in 1961 manually isolated the carboxyl position of amino acids via the ninhydrin reaction, and showed the carboxyl group to be enriched relative to the rest of the molecule. They further showed indirect evidence that glycolysis proceeds without isotope discrimination. Almost a decade passed before any further work appeared. A classic article on isotope fractionation established the precise enzymatic step at which acetate carbon is fractionated, resulting in the well-known depletion of ^{13}C in lipids (DeNiro & Epstein, 1977). Pyruvate oxidation to acetate was accomplished by procedures to isolate the carboxyl and methyl carbons of acetate for conventional bulk analysis. Results showed that the isotopic composition of these two positions were very different and that a kinetic isotope effect that discriminates against ^{13}C at the acetate carbon is the depleted site. These results predict alternating ^{13}C abundances along fatty acid aliphatic chains. That prediction has been verified experimentally (Melzer & Schmidt, 1987; Mouson & Hayes, 1982a, 1982b). In related work, Hayes and coworkers analyzed the ^{13}C content of the carboxyl and olefinic C in mono-unsaturated fatty acids from microorganisms (*E. coli* and *S. cerevisiae*), and the refined models of fatty acid metabolism that are based on their position-specific isotopic results (Monson & Hayes, 1982a; 1982b; Volger & Hayes, 1980; Monson & Hayes, 1980). These studies required Schmidt decarboxylation and oxidative ozonolysis to isolate specific carbon positions prior to high-precision determination. A representative review of research up until 1985 can be found (Galimov, 1985), including a discussion of the Galimov theory of biological isotopic fractionation. More recently, Ivlev and coworkers have discussed the relationship between isotopic fractionation in photosynthetic cells and the order of amino acid synthesis (Ivlev, 1986). Most recently, Weilacher et al. investigated intramolecular isotope ratios of C in several positions of caffeine and theobromine in order to study one-carbon metabolism (Weilacher, Gleixner, & Schmidt, 1996). In natural samples, they report a higher enrichment in fused-ring positions 2 and 8, which originate from the tetrahydrofolate pool, than in methyl groups that derive from S-adenosylmethionine ($\delta^{13}\text{C}$: -21.7‰ vs. -39‰); results from chemically synthesized samples showed much smaller average differences. In total, the body of scientific work on

the intramolecular distribution of carbon isotopes clearly indicates that they are indicative of a variety of important physiological processes, and might be routinely analyzed if an instrument were available to do so conveniently. Such an instrument would also facilitate many tracer experiments that are presently accomplished with high enrichments and analyzed using either NMR or organic MS. Until recently, high-precision position-specific measurements were at a similar stage as was CSIA prior to the mid-1970s, and required manual isolation of carbon positions within a molecule prior to analysis. That process is labor-intensive and practically impossible for determining many internal positions (Ivlev, 1991).

The bulk of recent work on pyrolysis-GC has applied the analytical capabilities of GC to nonvolatile, usually intractable, solids, such as high-molecular-weight polymer resins, keragens (Eglinton, 1994), and bacteria. Previously, several groups reported vapor-phase pyrolysis of small organic molecules for qualitative analysis as a possible alternative for GC-MS-based analysis (Dhout, 1961; 1963; Levy & Paul, 1967; Levy, 1967; Fanter, Walker, & Wolf, 1968; Walker & Wolf, 1968). These early studies showed that organic molecules of biological and commercial interest fragmented in a useful and characteristic way, and did so according to predictions of simple free radical fragmentation mechanisms that were elucidated decades earlier for hydrocarbons (Kossiakoff & Rice, 1943; Fabuss, Smith, & Satterfield, 1964). Although pyrolysis-GC systems never overshadowed GC-MS, they suggest a convenient and powerful means for fragmenting organic molecules prior to separation and analysis by IRMS, thereby providing an on-line strategy for the determination of isotope ratios from either groups or individual positions within molecules.

We have recently introduced a strategy for on-line PSIA, based on (1) pyrolytic fragmentation of analyte molecules, (2) measurement of fragment isotope ratios, and (3) calculation of isotope ratios for specific positions and/or for moieties (Corso & Brenna, 1997). Although there is considerable work involving the generation of isotope ratios from pyrolysis products (Mycke, Hall, & Leplat, 1994), there are little data on the prospects for generating reproducible isotope ratios from on-line pyrolysis in a microreactor. We have constructed test systems to evaluate this question for pentane and methyl palmitate (Me16:0).

The initial test system did not allow for separation prior to pyrolysis and admitted injected analyte into a pyrolysis furnace. *Headspace* pentane (about $10\text{ }\mu\text{L}$, $<300\text{ ng}$) was injected into the pyrolysis chamber at temperatures ranging from $100\text{--}500^\circ\text{C}$. Pyrograms and results are shown in Fig. 12 and Table 2. Below about 380°C , no fragmentation is observed. At 420°C , partial pyrolysis is observed, and the products are strong, reproducible peaks, as shown in Fig. 12(b). Figure 12(d) shows results at

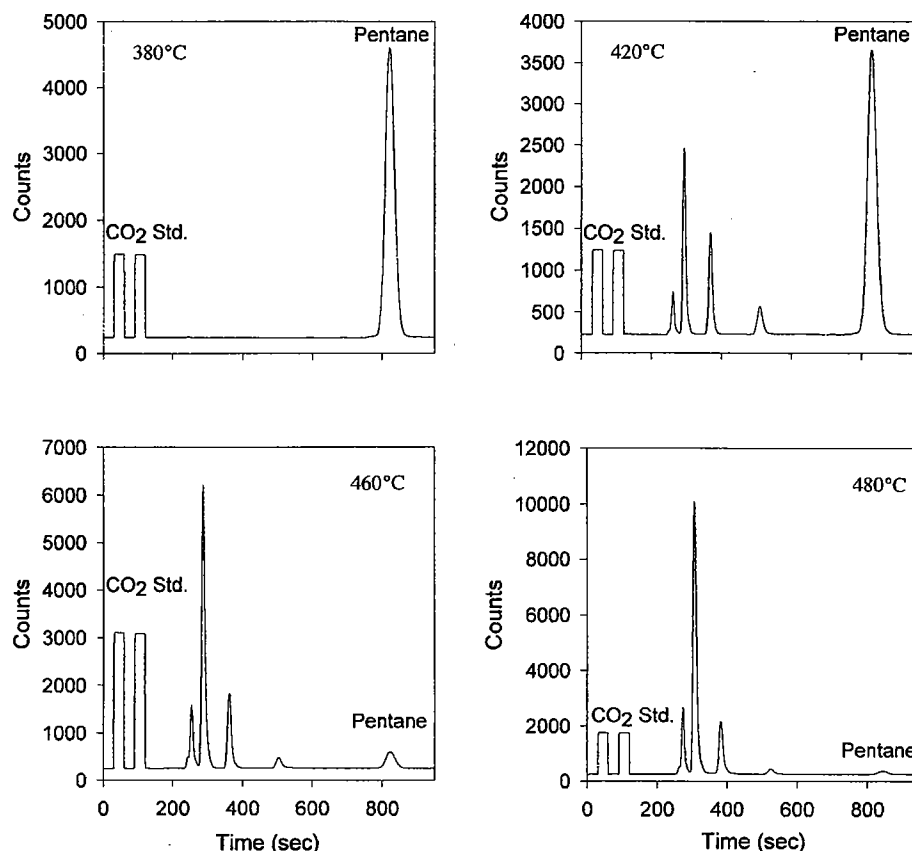


FIGURE 12. Results from analysis of pentane by PSIA at various pyrolytic temperatures. As the temperature is increased, pentane undergoes increasing degrees of fragmentation. Four fragment peaks are observed, corresponding to C1, C2, C3, and C4. Isotope ratios derived from these peaks are shown in Table 2 to be highly precise.

480°C, where pentane is almost quantitatively pyrolyzed and the products are the major peaks in the spectrum. Assuming no rearrangements, the four fragments correspond to C1, C2, C3, and C4, eluting in that order.

Table 2 presents the results of replicate analyses of the pyrolysis fragment peaks at 420°C. These data show that on-line pyrolysis is highly reproducible and that the

subsequent chromatography has sufficient quality for IRMS. The average precision is $SD(\delta^{13}C) < 1\%$, and is, therefore, sufficient to follow isotope ratio changes relevant in nature, even with this preliminary system. Some of the mean isotope ratios among the fragments are statistically different, indicating that the system is sensitive to changes in the isotope ratio. Methane is often observed to be the isotopically lightest hydrocarbon in nature. Progressively larger hydrocarbon chains increase in ^{13}C content up to C₃ or so (Baylis, Hall, & Jumeau, 1994).

Because this system demonstrated on-line pyrolysis to be reproducible, we constructed a second system, shown in Fig. 13, with a front end very similar to that of Levy and Paul in a tandem GC configuration (Levy & Paul, 1967). The principal additions to the previous system were: (1) the addition of a separation GC with appropriate valving to select a single purified peak into the pyrolysis chamber, and (2) the addition of a valve in the second GC to permit separated fragments to be admitted to an organic MS for structure analysis of pyrolysis products to verify

TABLE 2. Precision of carbon isotope ratio determinations from pyrolytic fragments of pentane.

| Replicate | C ₁ | C ₂ | C ₃ | C ₄ | Pentane |
|-----------|----------------|----------------|----------------|----------------|---------|
| 1 | -36.15 | -31.39 | -29.02 | -28.15 | -28.32 |
| 2 | -35.38 | -30.15 | -28.08 | -26.18 | -27.07 |
| 3 | -35.34 | -29.99 | -27.70 | -25.25 | -27.01 |
| 4 | -34.38 | -29.77 | -26.67 | -26.67 | -27.25 |
| Mean | -35.31 | -30.33 | -27.87 | -26.56 | -27.41 |
| S.D. | 0.72 | 0.73 | 0.97 | 1.21 | 0.61 |

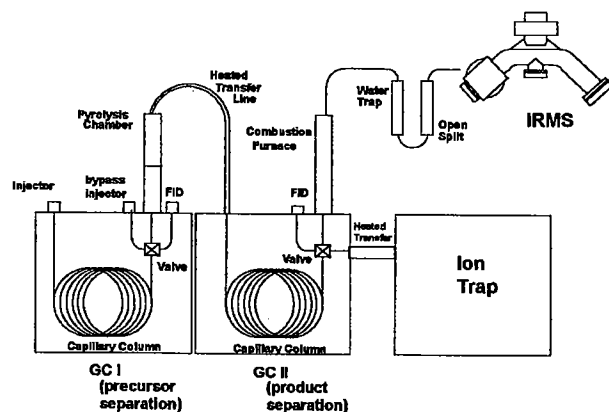


FIGURE 13. Diagram of a PSIA instrument that permits injection of a complex mixture into GC I, selection of a single peak into the pyrolysis furnace, separation of fragments in GC II, and analysis either of isotope ratio via combustion IRMS or structural analysis to identify fragments in an ion trap. (Reprinted with permission from PNAS 94:1049–1053, Fig. 1, February 1997, copyright 1997 National Academy of Sciences, U.S.A.)

structures predicted by theory and to perform labeling experiments to rule out any rearrangement. The test compound Me16:0 was purified in a first GC, and fragmented pyrolytically. The fragments were separated in the second GC, and were analyzed by either an ion trap MS, or an IRMS for high-precision isotope ratio analysis. Pyrolysis of Me16:0 yields two series of fragments that differ by one C up to the C₁₇ parent; structures are shown in Fig. 14. Data from labeling studies using [1-¹³C], [Me-¹³C], [16-¹³C], and [Me, 16-¹³C]-Me16:0 indicate no rearrangement of carbon due to the pyrolysis process. The difference in ¹³C atom fraction between consecutive fragments corresponds to the ¹³C atom fraction of the additional carbon position in the larger molecule, and can be calculated as a weighted difference between the two fragments. The reproducibility of fragment isotope ratios is SD(δ¹³C) = 0.3‰, and calculations of the isotope ratio of the carboxyl carbon were in good agreement with dilution data.

Data obtained for fragmentation at different temperatures showed that pyrolysis-induced fractionation was nearly insensitive to temperature. This observation leads directly to the conclusion that pyrolysis conducted at a single temperature should result in fragments with isotope ratios that are readily calibrated against standards. Results thus far indicate that controlled pyrolysis can be conducted on-line and can produce single bond breakage, stabilization, and isotopically representative fragments of carbon. The absence of any measurable rearrangement or major fractionation shows the approach to be applicable to position-specific studies of carbon at natural abundance or very low enrichment. Both of the systems that we have reported should be useful for PSIA, with the simpler system supe-

rior for purified small molecules, where fragmentation is known or is relatively simple; and the more complex system is appropriate for complex mixtures and larger, uncharacterized molecules.

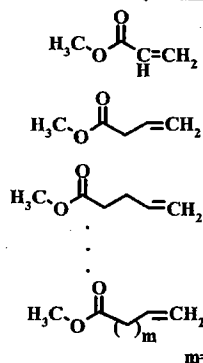
VI. NOTATION AND ELEMENTARY CALCULATIONS

Because the historical development of CF-IRMS focused on isotope variability due to natural processes, the notation and conventions used in this field derive from and apply to the long history of non-CSIA based on the dual inlet, which typically deals with variability over a very limited range of isotope ratios. As applications increase involving high enrichments characteristic of artificial isotope enrichment (Goodman & Brenna, 1992; Cunnane et al., 1995; Carnielli et al., 1996; Brenna, 1997) or using very low enrichment compounds derived naturally (Tissot et al., 1990; Dammelmair et al., 1995), there is a need to review basic relationships. In this section, we bring together notations and relationships used in natural variability research coupled to relationships derived to calculate useful quantities for tracer applications. For simplicity, we consider the case of C, but all equations apply directly to other elements with simple substitution.

A. Delta Notation

The delta notation was first formally defined by McKinney et al. (McKinney et al., 1950; Hayes, 1983), and is the

ω-Monounsaturated Methyl Ester Series



α-Olefin Series

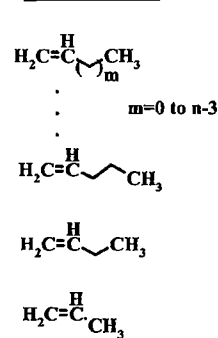


FIGURE 14. Structure of stable pyrolytic fragments from methyl palmitate. The hydrocarbon chain breaks between each C—C bond, starting at C3—C4. Unsaturation is introduced into each fragment by ejection or rearrangement of H. There is no detectable scrambling of C in the cleavage or stabilization process as predicted for the free radical-mediated mechanism, although rearrangement is predicted for the hydrogens. These fragments are isotopically representative of the original molecular isotope distribution. (Reprinted with permission from PNAS 94:1049–1053, Fig. 3, February 1997, copyright 1997 National Academy of Sciences, U.S.A.)

conventional means for expressing high-precision isotope ratios. It is defined as the relative difference in parts per thousand between the sample isotope ratio and an isotope ratio of an international standard. In the case of C, the standard is PeeDee Belemnite (PDB), expressed as

$$\delta^{13}\text{C}_{\text{PDB}} = \left(\frac{R_{\text{STD}} - R_{\text{PDB}}}{R_{\text{PDB}}} \right) \times 1000 = \left(\frac{R_{\text{SPL}}}{R_{\text{PDB}}} - 1 \right) \times 1000 \quad (8)$$

with

$$R_x = \frac{[^{13}\text{C}]}{[^{12}\text{C}]}, \quad (9)$$

where $[^{13}\text{C}]$ and $[^{12}\text{C}]$ are the abundances of the respective isotopes in the sample or PDB, $R_{\text{PDB}} = 0.0112372 \pm 0.0000090$ (Craig, 1957), and where the latter $\delta^{13}\text{C}_{\text{PDB}}$ form is more convenient for calculations. In practice, abundances from samples and working standards calibrated against PDB are used to calculate δ_{WS} , and the following form is used to convert δ_{WS} to $\delta^{13}\text{C}_{\text{PDB}}$:

$$\delta_{\text{PDB}}^{\text{SPL}} = \delta_{\text{WS}}^{\text{PDB}} + \delta_{\text{SPL}}^{\text{WS}} + \frac{\delta_{\text{PDB}}^{\text{PDB}} \delta_{\text{SPL}}^{\text{WS}}}{1000}, \quad (10)$$

where superscripts refer to the reference ratio. A very useful brief discussion of international standards is available (Ehleringer & Rundel, 1988).

It is frequently necessary to discuss the precision associated with IRMS measurements, often in the context of analytical development. For this purpose, we use $\text{SD}(\delta^{13}\text{C})$ as the standard deviation (root mean square deviation) expressed in $\delta^{13}\text{C}$ units.

B. Atom Fraction (Percent) Notation

In tracer studies, it is more convenient to express isotope data in terms of isotope concentrations rather than the relative ratio form of $\delta^{13}\text{C}$. For high-precision results, it is first necessary to calculate $\delta^{13}\text{C}$ from ion current ratios of sample and standard, from which calibrated isotope ratios, R_x , can be extracted. Atom fractions (F_x) and atom percents (AP) can be calculated according to the following:

$$F = \frac{^{13}\text{C}}{^{12}\text{C} + ^{13}\text{C}} = \frac{R_s}{1 + R_s}; \quad AP = F \times 100. \quad (11)$$

Atom percent excess (APE) is expressed in two different ways:

$$APE = (AP)_E - (AP)_B \quad (12)$$

OR

$$APE' = \frac{R_E - R_B}{1 + (R_E - R_B)} \times 100. \quad (12b)$$

By definition, APE is given by the difference between AP for enriched samples and AP at background (Coleman & Fry, 1992), and reflects the percentage of labeled atoms in excess of natural background. The alternative formulation (APE') is widely used (Wolfe, 1992), but the quantities are not analytically equivalent, even though they yield similar values near natural abundance.

To illustrate the difference between the two formulations for APE and other means for expressing isotope concentration, we consider a range of enrichments from natural abundance to near 100% ^{13}C as shown in Table 3. The sixth column shows that the difference between APE and APE' is experimentally very small near natural abundance. At high enrichment, APE approaches $98.8888 = 100 - F_{\text{PDB}}$, and APE' approaches 100. Thus, APE' expresses the percentage between baseline and maximal enrichment, so that a compound with 100% ^{13}C is equivalently expressed as $APE' = 100$. Table 3 also shows how $\delta^{13}\text{C}$ approaches infinity at high enrichments, making it very cumbersome for calculations involving highly enriched tracers. To preserve most of the advantages of this notation, we have proposed (Corso & Brenna, 1997) an alternative formulation based on isotope fractions as follows:

$$\begin{aligned} \phi^{13}\text{C}_{\text{PDB}} &= \left(\frac{F_{\text{SPL}} - F_{\text{PDB}}}{F_{\text{PDB}}} \right) \times 1000 \\ &= \left(\frac{F_{\text{SPL}}}{F_{\text{PDB}}} - 1 \right) \times 1000. \quad (13) \end{aligned}$$

This notation has the advantage that its units are nearly identical to the common $\delta^{13}\text{C}$, relative parts per thousand, and increasing linearly up to 100% ^{13}C . The last two columns of Table 3 show $\phi^{13}\text{C}$ and the difference between $\delta^{13}\text{C}$ and $\phi^{13}\text{C}$ around natural abundance and at high enrichments. Near natural abundance, the difference is sufficiently small so that it will be discernible only with very precise CF measurements that are somewhat removed from the isotope ratio of the international reference standard. At highest enrichments, $\phi^{13}\text{C}$ rises linearly rather than approaching infinity like $\delta^{13}\text{C}$. The major advantage of $\phi^{13}\text{C}$ becomes apparent in calculations involving mixing of isotope pools requiring the mass balance equation.

TABLE 3. Comparison of various notations to express high-precision isotope data, using carbon as an example.

| $\delta^{13}\text{C}$ (‰) | R | F | APE | APE' | $APE-APE'$ | $\phi^{13}\text{C}$ (‰) | $\delta-\phi$ |
|---------------------------|--------|--------|---------|---------|------------|-------------------------|---------------|
| -30 | 0.0109 | 0.0108 | -0.0330 | -0.0337 | 0.0007 | -29.6765 | -0.3235 |
| 0 | 0.0112 | 0.0111 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 |
| 50 | 0.0118 | 0.0117 | 0.0549 | 0.0562 | -0.0012 | 49.41 | 0.58 |
| 1000 | 0.0225 | 0.0220 | 1.0868 | 1.1112 | -0.0244 | 978 | 22 |
| 354,961 | 4.0000 | 0.8000 | 78.8888 | 79.9549 | -1.0662 | 79,992 | 283,968 |
| 8,809,024 | 99 | 0.9900 | 97.8888 | 98.9999 | -1.1111 | 88,090 | 8,720,934 |
| 89,900,150 | 999 | 0.9990 | 98.7888 | 99.9000 | -1.1112 | 88,900 | 88,811,250 |
| 889,811,409 | 9999 | 0.9999 | 98.8788 | 99.9900 | -1.1112 | 88,981 | 889,722,428 |

C. Mass Balance

The fundamental relationship used for many years in high-precision work to determine relative contributions of two or more carbon sources to a pool is mass balance, also referred to as the *Master Equation*. This two-part equation is often cast as follows:

$$\delta_A m_A + \delta_B m_B = \delta_C m_C \quad (14)$$

$$m_A + m_B = m_C \quad (15)$$

Substituting the definition of δ into (14) gives

$$\frac{R_A - R_{PDB}}{R_{PDB}} m_A + \frac{R_B - R_{PDB}}{R_{PDB}} m_B = \frac{R_C - R_{PDB}}{R_{PDB}} m_C \quad (16)$$

Collecting terms, we have

$$R_A m_A + R_B m_B = R_C + R_{PDB}[m_A + m_B - m_C] \quad (17)$$

Noting that the bracketed term equals 0 from (15), we have

$$R_A m_A + R_B m_B = R_C m_C \quad (18)$$

which demonstrates that (14) is equivalent to a weighted sum of isotope ratios. This relation is not analytically correct, as is evident from the cross-term of (10). It is isotope concentrations, or conventionally expressed atom fractions or atom percents, to which mass balance applies. We have

$$F_A m_A + F_B m_B = F_C m_C \quad (19)$$

where F_x is the atom fraction of "x" given by

$$F_x = \frac{[^{13}\text{C}]}{[^{12}\text{C}] + [^{13}\text{C}]}; \quad F = \frac{R}{1 + R} \quad (20)$$

In the neighborhood of natural abundance for carbon, the error introduced by using (14) instead of (20) is negli-

gible, as evidenced by the common use of expressions of "delta-over-baseline," $DOB = \delta_{\text{ENRICHED}} - \delta_{\text{BASELINE}}$. However, for studies involving enriched material, R results in large deviations as enrichment goes to $^{13}\text{C} = 100\%$. We can also cast an analytically correct mass balance equation in *per mil* (‰) using $\phi^{13}\text{C}$, as follows:

$$\phi^{13}\text{C}_a m_a + \phi^{13}\text{C}_b m_b = \phi^{13}\text{C}_c m_c \quad (21)$$

This equation directly yields analytically correct isotope concentrations in parts per thousand units without cross-terms and without resort to conversions after manipulations. For simplicity and to follow convention, we will use Eq. (19); however, all results can be easily adapted to the $\phi^{13}\text{C}$ notation.

D. Tracer Studies Using High-Precision CSIA

There is an increasing interest in the use of high-precision CSIA in tracer studies. Several years ago, we demonstrated that increasing enrichment of precursor compounds produces significantly improved detection limits, with highest enrichment, uniformly labeled compounds producing the highest sensitivity (Goodman & Brenna, 1992), unlike organic GC/MS where increasing label has no general effect on detection limits.

Detection limits are defined for most analytical techniques in terms of a particular threshold S/N ratio obtained upon analysis of a minimal amount of sample, with units often expressed as mean/SD. High-precision implies measurement of strong signals; thus, detection limits in these terms are seldom if ever quoted for IRMS instruments. Rather, there is a minimal sample size required to yield a threshold precision, and sample size is quoted on a per mol or per gram element basis. For example, analytical performance for CF carbon analysis might be quoted as 10 ng C to produce a precision of $SD(\delta^{13}\text{C}) < 0.3\%$.

Tracer detection limits can be defined in terms of the least significant difference (LSD) above baseline that can be declared significant, as has been discussed previously

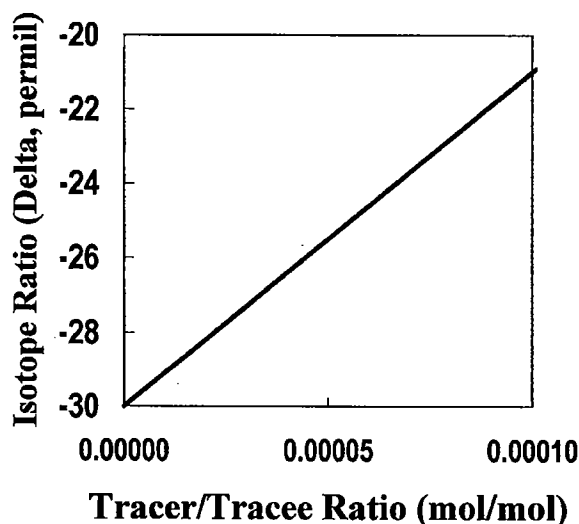


FIGURE 15. Theoretical relationship between tracer-tracee ratio and measured $\delta^{13}\text{C}$, calculated assuming an endogenous value of $\delta^{13}\text{C} = -30\text{‰}$ and a tracer ^{13}C atom fraction = 1, using the mass balance equation. This plot shows the increase in isotope ratio expected with increasing tracer-tracee ratio. If the analytical precision, including all workup steps, is no worse than $\text{SD}(\delta^{13}\text{C}) < 0.5\text{‰}$ and the baseline does not contribute appreciable variability, then dilutions of up to 10^5 can be detected by this tracer technique.

(Brenna, 1994). As in any analytical technique, the LSD depends on the measurement precision, which in turn depends on sample size. Choosing a sample size of one nmol carbon, which should easily produce a precision of $\text{SD}(\delta^{13}\text{C}) = 0.4\text{‰}$, we use mass balance to give a plot of $\delta^{13}\text{C}$ vs. the tracer:tracee ratio (T/T) in any pool. To construct the plot shown in Fig. 15, we assume that the endogenous compound has an isotope ratio corresponding to $\delta^{13}\text{C} = -30\text{‰}$, and that the labeled compound is uniformly labeled with an atom fraction $F = 1$. The plot shows that a 1‰ increase above baseline corresponds approximately to a 1:100,000 dilution, indicating that this value is the limiting dilution for high-precision measurements.

It is worth noting that the background variability depends on the external precision of the instrument and on the natural variability of the backgrounds under study. For instance, the external precision of GCC-IRMS can reliably be obtained at a level of $\text{SD}(\delta^{13}\text{C}) < 0.4\text{‰}$, which sets a lower bound on detectability. However, the two photosynthetic pathways, commonly designated C3 and C4, fractionate carbon isotopes to differing degrees, resulting in differences of about 20‰ in materials derived from these two sources. Isotope ratio measurements in animals whose source of carbon is ultimately derived from plants using both photosynthetic pathways will vary in a complex way between these extremes. Instrumental external precision in these experiments may be a minor factor affecting detection limits if diet is variable and uncontrolled.

E. Mass Balance: Pool Mixing

High-precision is most easily achieved near natural abundance for a variety of reasons, including similarity of peak-shapes among the various detection channels and closeness for calibration against standards. For this reason, it is necessary to dilute highly enriched dose material to facilitate high-precision determination. The mass balance equation for dilution of a chemically pure, highly enriched species with the chemically identical natural abundance species is

$$F_N m_N + F_L m_L = F_T m_T; \quad m_E + m_L = m_T, \quad (22)$$

where N refers to natural abundance diluent, L is a highly enriched labeled material, and T is total. We define dilution as

$$D = \frac{m_L}{m_N + m_L} = \frac{m_L}{m_T}; \quad m_L = D m_T. \quad (23)$$

We wish to plot F_T vs. D , so

$$F_T = F_N \left(\frac{m_N}{m_T} \right) + F_L \left(\frac{m_L}{m_T} \right) \quad (24)$$

$$F_T = F_N(1 - D) + F_L D \quad (25)$$

$$F_T = F_N + D(F_L - F_N). \quad (26)$$

Equation (26) shows that a plot of F_T vs. D yields a y-intercept of F_N and a slope of $F_L - F_N$. It further shows that the concentration of the original solutions is not required for these calculations. Errors associated with the determination of F_N and F_L depend only on the determination of F_T and the quality of quantitative volume dilutions.

The last point is worthy of emphasis. The precision and accuracy of F_L determination is limited by the precision and accuracy of the quantitative dilution. Because highly enriched samples are difficult to analyze precisely by IRMS (or other means), there is the temptation to dilute with natural abundance material to bring the isotope ratio into an easily measured range. However, the precision of the IRMS measurement will be lost if the dilution is not of the same precision. An example is the dilution of a body fluid, for instance milk, originally with a $\delta D > 2000\text{‰}$. Milk is highly variable in its solute content, which conversely can be thought of as being highly variable in its water content. Simple gravimetric dilution with water of known isotope ratio will not yield a high-precision dilution factor, because the weight of water in the milk is not known. Weight of water can be characterized with precision required for D analysis (Brenna & Yeager, 1995) with coefficients of variation consistently less than 0.1% by gravimetric determination of weight loss after very careful drying. However, this precision may not be suffi-

cient to preserve precision for $\delta^{18}\text{O}$ measurements. Drying also does not take into account the presence of volatiles that are not in isotopic equilibrium with the analyte.

Introduction of labeled molecules into an endogenous pool results in elevated isotope ratio. If there is no covalent modification of the tracer, and no labeled atoms are displaced or altered in isotope ratio, then the mass balance equation to describe this process takes the form:

$$F_T m_T = F_L m_L + F_E m_E; \quad m_T = m_L + m_E \quad (27)$$

or

$$F_T = F_L \left(\frac{m_L}{m_T} \right) + F_E \left(\frac{m_E}{m_T} \right) \quad (28)$$

Because

$$\frac{m_E + m_L}{m_T} = 1 \quad (29)$$

we have

$$F_T = F_L \left(\frac{m_L}{m_T} \right) + F_N \left(1 - \frac{m_L}{m_T} \right), \quad (30)$$

where we have replaced the subscript "N" with "E" to refer to *endogenous*. Rearranging yields the final result,

$$\frac{m_L}{m_T} = \frac{F_T - F_E}{F_L - F_E} = \frac{AFE}{F_L - F_E}, \quad (31)$$

where $F_T - F_E$ has been replaced by atom fraction excess (AFE). Equation (22) indicates that the ratio of tracer to total material (tracer:tracee ratio, " T/T ") is given by the ratio of the difference in enrichment between the tracer and endogenous divided by the difference between the final and endogenous enrichments. When $F_L = 1$, as it does for highly enriched compounds, to a good approximation $F_L \gg F_E$, $F_L - F_E \approx F_L = 1$ and

$$\frac{m_L}{m_T} = AFE = \frac{APE}{100}, \quad (32)$$

which shows that the tracer:tracee ratio is approximated by the atom fraction enrichment, or equivalently the atom percent enrichment divided by 100.

In tracer experiments, it will often be desirable to calculate the quantity of a product derived from a different tracee compound. The situation is most easily illustrated by means of an example. Fatty acids play critical roles in many physiological processes. The level of interconver-

sion of fatty acids from dietary precursors is frequently of importance to determine adequate dietary levels and partitioning between pathways. Consider the case of the conversion of α -linolenic acid ($\text{C}_{18}\text{H}_{30}\text{O}_2$, "18:3"), a straight chain triunsaturated fatty acid, to the product docosahexaenoic acid ($\text{C}_{22}\text{H}_{32}\text{O}_2$, "22:6") by the addition of four carbons and the introduction of three additional double bonds. A dose of highly enriched 18:3 is metabolized to 22:6 by the addition of 4 carbons whose isotope ratio is in the range of the endogenous 22:6. Hence, the isotope ratio of labeled 22:6 will be lower than the dose with this endogenous dilution by a factor of 4/22, and the application of the previous equations to determine T/T will result in an error of this magnitude. We can derive an expression to account for this dilution using mass balance to arrive at the total mol of labeled 22:6 molecules. The mass balance equation takes the form:

$$F_T m_T = F_E m_E + m_L (\alpha F_L + \beta F_E); \quad m_T = m_E + m_L; \quad \alpha + \beta = 1, \quad (33)$$

where the F_T , m_T , F_E , and m_E are defined for the product, and F_L is the atom fraction of the *precursor*, because it refers to the labeled carbons. α is the number of mol of carbon per mol precursor compound divided by the mol carbon per mol product. In our example, there are 18 mol of C per mol 18:3, 22 mol C per mol 22:6, so $\alpha = 18/22$. β can be interpreted as the mol of endogenous carbon added to each mol of labeled product; in our example, 4/22. $F_L = 1$ if 18:3 is 100% ^{13}C . Dividing the equation by m_T , collecting terms, and solving for T/T , we have

$$\frac{m_L}{m_T} = \frac{F_T - F_E}{\alpha F_L + \beta F_E - F_E} = \frac{F_T - F_E}{\alpha F_L + F_E(\beta - 1)}. \quad (34)$$

Recognizing that $\alpha + \beta = 1$ can be rearranged to yield $\beta - 1 = -\alpha$, substituting, and collecting terms, we arrive at the final result:

$$\frac{m_L}{m_T} = \frac{1}{\alpha} \left(\frac{F_T - F_E}{F_L - F_E} \right) = \frac{1}{\alpha} \left(\frac{AFE}{F_L - F_E} \right). \quad (35)$$

This equation is identical to the T/T expression in Eq. (31), with $1/\alpha$ serving as a proportionality constant. In our example, m_L/m_T is the molar fraction of 22:6 that is labeled with 18:3. If all the 22:6 were derived from 18:3, and 18 of each 22 carbons were labeled, then $m_L/m_T = 1$.

Because CSIA does not detect isotopomers, it is not possible to distinguish between product molecules resulting from simple carbon addition to the precursor from molecules labeled by catabolism and *de novo* synthesis. In our example, it is thought that 18:3 oxidation to acetate, and subsequent synthesis to fatty acids and other com-

pounds is an important physiological mechanism for the brain. Although 22:6 cannot be synthesized from acetate, labeled acetate could be used for elongation of endogenous 18:3 to yield 22:6 labeled in only two positions rather than 18. This phenomenon is observed for the synthesis of saturated fatty acids from a number of unsaturated precursors (Sheaff et al., 1996). Hence, T/T in this context can be best thought of as “dose-equivalents.” Context will often suggest a most prominent form so that little ambiguity may remain.

F. Dilution Space

Calculation of dilution space requires the determination of the initial enrichment of the dose. For tracer experiments, the dose enrichment is very high compared to the endogenous pool and must, in general, be diluted prior to high-precision determination.

Two mass-balance equations can be used to derive the relationship between the relevant enrichments and the weights of the doses and diluents. The mass-balance equation relating dose and body pools is

$$F_T m_T = F_L m_L + F_E m_E, \quad (36)$$

where the subscripts T , L , and E refer to *Total*, *Label*, and *endogenous*, respectively. The mass-balance equation for the dilution of an aliquot of dose with natural abundance material can be written:

$$F_{dD} m_{dD} = F_D m'_D + F_d m_d \quad (37)$$

with the subscripts dD , D , and d referring to *diluted Dose*, *Dose*, and *diluent*, respectively, and the prime of m_D distinguishes the quantity of dose used for dilution from that of Eq. (36). If we make the assumption that $m_T \sim m_E \gg m_L$ in (36) and that m_{dD} approximates m_d in (37), then we can replace m_T with m_E in (36) and m_d with m_{dD} in (37). Rearranging (37) we have

$$F_D = \frac{m_{dD}(F_{dD} - F_d)}{m'_D}. \quad (38)$$

Substituting F_D into (36), and recognizing that $m_D = W_D/M_D$, where W is weight and M is the molecular weight, we have

$$F_T m_e = \left(\frac{m_{dD}(F_{dD} - F_d)}{(W'_D/M_D)} \right) \left(\frac{W_D}{M_D} \right) + F_e m_e, \quad (39)$$

with M_D canceling. Rearranging, we find

TABLE 4. Chronology of selected developments in Continuous Flow (CF) IRMS instrumentation.

| Authors (citation) | Element ^b |
|---|----------------------|
| CF ^a | |
| Sano et al. [1976] | C |
| Matthews & Hayes [1978] | C, N |
| Markey & Abramson [1982] | C |
| Elemental Analyzer/Bulk Analysis ^c | |
| Preston [1983] | N |
| Preston [1985] | C |
| Giesemann et al. [1994] | S |
| Brand, Tegtmeier, & Hilkert [1994] | O |
| Prosser & Scrimgeour [1995] | H |
| Tobias et al. [1995] | H |
| GCC-IRMS ^d | |
| Barrie, Bricout, & Koziet [1984] | C |
| Preston & Slater [1994] | N |
| Merritt & Hayes [1994] | N |
| Tobias & Brenna [1996] | H |
| LCC-IRMS ^d | |
| Caimi & Brenna [1993] | C |
| Teffera, Kuzmierz, & Abramson [1996] | N, (C) |
| PSIA | |
| Corso & Brenna [1997] | C |

^a Original work coupling GC separation to online chemical treatment to yield CO₂ or N₂ to be analyzed by single collector mass spectrometers.

^b Refers to element reported.

^c CF-IRMS with no sample chemical separation capability.

^d CF-IRMS with separation step and multidetector instrument.

$$m_e = \frac{F_{dD} - F_d}{F_T - F_E} \left(\frac{W_D}{W'_D} \right) \left(\frac{W_{dD}}{M_{dD}} \right). \quad (40)$$

This final equation yields the very convenient result that the molecular weight of the highly enriched dose cancels. This relationship is very convenient, because the molecular weight for an enriched compound in general cannot be calculated from natural abundances because the abundance of “unlabeled” elements may be affected by the isotope purification procedures. The dilution space depends on the relative enrichments of the body pool and the dilution pool, the weights of the enriched material in each space, and the diluent. The inverse of this equation can also be viewed as a scaling of the enrichment by the dose level, and is particularly convenient for plotting more than one isotope enrichment on the same scale, as has been recommended by Coward for doubly labeled water studies (Prentice, 1988).

VII. CONCLUSIONS

In Table 4, we have compiled a chronology of developments in CF-IRMS for reference and to illustrate the state-of-the-science. There remain many fundamental instrumentation advances, most notably development leading to the routine analysis of H isotopes after chromatographic separation, the routine application of liquid separation-based techniques to IRMS, and the application of PSIA to detect intramolecular variations in isotope ratio. The CF techniques now routinely solving application problems are elemental analyzer determination of C and N, GC applications for C, analysis of isotopes in water using the equilibration techniques, CO₂ breath analysis, and trace gas analysis. Additional penetration into the myriad of applications for IRMS awaits instrument refinements and commercialization. Continuing improvements in precision and sensitivity of CF-IRMS, along with automation, expand its applicability and ensure that it continues as an important and unique tool of analytical mass spectrometry.

ACKNOWLEDGMENTS

We acknowledge many helpful discussions with Keith Goodman, Martin Schoell, Leah Houghton, Simon Prosser, Paul Haggarty, and Charles Douthitt.

REFERENCES

1. Abelson, P. H.; Hoering, T. C. "Carbon isotope fractionation in formation of amino acids by photosynthetic organisms," *Proc. Nat. Acad. Sci. USA* **1961**, *47*, 623–632.
2. Abramson, F. P.; Teffera, Y.; Kusmierz, J.; Steenwyk, R. C.; Pearson, P. G. "Replacing ¹⁴C with stable isotope in drug metabolism studies," *Drug Metab. Disposition* **1996**, *24*, 697–701.
3. Aggarwal, P. K.; Fuller, M. E.; Gurgas, M. M.; Manning, J. F.; Dillon, M. A. "Use of stable oxygen and carbon isotope analyses for monitoring the pathways and rates of intrinsic and enhanced *in situ* biodegradation," *Environ. Sci. Technol.* **1997**, *31*, 590–596.
4. Aston, F. W. *Mass Spectra and Isotopes*, Longmans, Green, and Co: New York, 1942.
5. Barrie, A.; Bricout, J.; Koziat, J. "Gas chromatography-stable isotope ratio analysis at natural abundance levels," *Biomed. Mass Spectrom.* **1984**, *11*, 583–588.
6. Baylis, S. A.; Hall, K.; Jumeau, E. J. "The analysis of the C1-C5 components of natural gas samples using gas chromatography-combustion-isotope ratio mass spectrometry," *Org. Geochem.* **1994**, *21*, 777–785.
7. Begley, I. S.; Scrimgeour, C. M. "On-line reduction of H₂O for $\delta^2\text{H}$ and $\delta^{18}\text{O}$ measurement by continuous-flow isotope ratio mass spectrometry," *Rap. Comm. Mass. Spectrom.* **1996**, *10*, 969–973.
8. Begley, I.; Scrimgeour, C. "High-precision δD and $\delta^{18}\text{O}$ measurement for water and volatile organic compounds by continuous-flow pyrolysis isotope ratio mass spectrometry," *Anal. Chem.* **1997**, *69*, 1530–1535.
9. Bocek, P.; Deml, M.; Tesarik, K. J. "The determination of ²H₂O in water and biological fluids by gas chromatography," *J. Chromatogr.* **1973**, *87*, 246–249.
10. Boutton, T. W.; Wong, W. W.; Hachey, D. L.; Lee, S. L.; Cabrera, M. P.; Klein, P. D. "Comparison of quartz and pyrex tubes for combustion of organic samples for stable carbon isotope analysis," *Anal. Chem.* **1983**, *55*, 1832–1833.
11. Brand, W. A. "Precon: A fully automated interface for the pre-GC concentration of trace gases in air for isotopic analysis," *Isotopes Environ. Health Stud.* **1995**, *31*, 277–284.
12. Brand, W. A. "High-precision isotope ratio monitoring techniques in mass spectrometry," *J. Mass Spec.* **1996**, *31*, 225–235.
13. Brand, W. A.; Tegtmeier, A. R.; Hilkert, A. "Compound-specific isotope analysis: extending toward ¹⁵N/¹⁴N and ¹⁸O/¹⁶O," *Org. Geochem.* **1994**, *21*, 585–594.
14. Brenna, J. T. "High-precision gas isotope ratio mass spectrometry: recent advances in instrumentation and biomedical applications," *Acc. Chem. Res.* **1994**, *27*, 340–346.
15. Brenna, J. T. "The use of stable isotopes to study fatty acid and lipoprotein metabolism in man," *Prostagl. Leuko. Ess. Fatty Acids*, to appear.
16. Brenna, J. T.; Yeager, K. E. "Direct determination of D in water and untreated urine by NMR: Application to doubly labeled water analysis," *Am. J. Physiol.* **1995**, *268 (Endocrinol Metab. 31)*, E1018–E1026.
17. Brenninkmeijer, C. A. M.; Mook, W. G. "A batch process for direct conversion of organic oxygen and water to CO₂ for ¹⁸O/¹⁶O Analysis," *Int. J. Appl. Radiat. Isot.* **1981**, *32*, 137–141.
18. Burlingame, A. L.; Boyd, R. K.; Gaskell, S. K. "Mass spectrometry," *Anal. Chem.* **1996**, *68*, 599R–651R.
19. Caimi, R. J. "The development and application of liquid chromatography-combustion-high precision isotope ratio mass spectrometry. Ph. D. Thesis, Cornell University: Ithaca, NY, 1995.
20. Caimi, R. J.; Brenna, J. T. "High precision liquid chromatography-combustion isotope ratio mass spectrometry (LCC-IRMS)," *Anal. Chem.* **1993**, *65*, 3497–3500.
21. Caimi, R. J.; Brenna, J. T. "Carbon isotope fractionation during reversed phase liquid chromatography," *J. Chromatogr.* **1995**, 307–310.
22. Caimi, R. J.; Houghton, L. H.; Brenna, J. T. "Condensed phase standards for high precision compound specific isotope analysis," *Anal. Chem.* **1994**, *66*, 2989–2991.
23. Carnielli, V. P.; Wattimena, D. J. L.; Luijendijk, I. H. T.; Boerlage, A.; Degenhart, H. J.; Sauer, P. J. J. "The very low birth weight premature infant is capable of synthesiz-

- ing arachidonic and docosahexaenoic acids from linoleic and linolenic acids," *Ped. Res.* **1996**, 169–174.
24. Chapman, T. E.; Berger, R.; Reijngoud, D. J.; Okken, A. *Stable Isotopes in Paediatric Nutritional and Metabolic Research*; Intercept: Andover, NH, 1990.
 25. Ciais, P.; Tans, P. P.; Trolier, M.; White, J. W. C.; Francey, R. J. "A large northern hemisphere terrestrial CO₂ sink indicated by the ¹³C/¹²C ratio of atmospheric CO₂," *Science* **1995**, 269, 1098–1102.
 26. Clarke, H. T. *The use of isotopes in biology and medicine*, The University of Wisconsin Press: Madison, WI, 1948.
 27. Cohn, M.; Urey, H. C. "Oxygen exchange reactions of organic compounds and water," *J. Am. Chem. Soc.* **1938**, 60, 679–683.
 28. Coleman, D. C.; Fry, B. *Carbon Isotope Techniques*; Academic Press: San Diego, 1991.
 29. Coleman, M. L.; Sheperd, T. J.; Durham, J. J.; Rouse, J. E.; Moore, G. R. "Reduction of water with zinc for hydrogen isotope analysis," *Anal. Chem.* **1982**, 54, 993–995.
 30. Conny, J. M.; Currie, L. A. "The isotopic characterization of methane, non-methane hydrocarbons and formaldehyde in the troposphere," *Atmos. Environ.* **1996**, 30, 621–638.
 31. Coplen, T. B. "Normalization of oxygen and hydrogen isotope data," *Chem. Geol.* **1988**, 72, 293–297.
 32. Coplen, T. B. "Editorial: More uncertainty than necessary," *Paleoceanography*, **1996**, 11.
 33. Coplen, T. B.; Wildman, J. D.; Chen, J. "Improvements in the gaseous hydrogen–water equilibration technique for hydrogen isotope ratio analysis," *Anal. Chem.* **1991**, 63, 910–912.
 34. Corso, T. N.; Brenna, J. T. "High-precision position-specific isotope analysis," *Proc. Natl. Acad. Sci. USA* **1997**, 94, 1049–1053.
 35. Craig, H. "The geochemistry of the stable carbon isotopes," *Geochim. Cosmochim. Acta* **1953**, 3, 53–92.
 36. Craig, H. "Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide," *Geochim. Cosmochim. Acta* **1957**, 12, 133–149.
 37. Cunnane, S. C.; Ryan, M. A.; Craig, K. S., et al. "Synthesis of linoleate and α -linolenate by chain elongation in the rat," *Lipids* **1995**, 30, 781–783.
 38. Dammelmair, H.; Schenck, U. V.; Behrendt, E.; Sauerwald, T.; Koletzko, B. "Estimation of arachidonic acid synthesis in full term neonates using natural variation in ¹³C content," *J. Pediatr. Gastro. Nutr.* **1995**, 21, 31–36.
 39. Dawson, T. E.; Ehleringer, J. R. "Gender-specific physiology, carbon isotope discrimination, and habitat distribution in boxelder, *Acer Negundo*," *Ecology* **1993**, 74, 798–815.
 40. DeNiro, M. J.; Epstein, S. "Mechanism of carbon isotope fractionation associated with lipid synthesis," *Science* **1977**, 261–263.
 41. Dhont, J. H. "Pyrolysis and gas chromatography for the detection of the benzene ring in organic compounds," *Nature* **1961**, 192, 747–748.
 42. Dhont, J. H. "Identification of organic compounds from food odours by pyrolysis," *Nature* **1963**, 200, 882.
 43. Eglinton, T. I. "Carbon isotopic evidence for the origin of macromolecular aliphatic structures in keragens," *Org. Geochem.* **1994**, 21, 721–735.
 44. Ehleringer, J. R.; Hall, A. E.; Farquhar, G. D. *Stable isotopes and plant carbon-water relations*; Academic Press, Inc.: San Diego, CA, 1993.
 45. Ehleringer, J. R.; Rundel, P. W. "Stable isotopes: history, units, and instrumentation. In *Stable Isotopes in Ecological Research*, Rundel, P. W.; Ehleringer, J. R.; Nagy, K. A., Eds.; Springer-Verlag: New York, 1988.
 46. Fabuss, B. M.; Smith, J. O.; Satterfield, C. N. "Thermal cracking of pure saturated hydrocarbons." In *Advances in Petroleum Chemistry and Refining*, Vol. 9; McKetta, J., Ed.; J. Wiley & Sons: New York, 1964.
 47. Fanter, D. L.; Walker, J. Q.; Wolf, C. J. "Pyrolysis-gas chromatography of hydrocarbons," *Anal. Chem.* **1968**, 40, 2168–2175.
 48. Farquhar, G. D.; Henry, B. K.; Styles, J. M. "A rapid on-line technique for determination of oxygen isotope composition of nitrogen-containing organic matter and water," *Rap. Comm. Mass Spectrom.* **1997**, 11, 1554–1560.
 49. Frazer, J. W. "Simultaneous determination of carbon, hydrogen and nitrogen. Part II," *Mikrochim. Acta* **1962**, 6, 993–999.
 50. Friedman, I. "Deuterium content of natural waters and other substances," *Geochim. Cosmochim. Acta* **1953**, 4, 89–103.
 51. Fry, B.; Garritt, R.; Tholke, F., et al. "Cryoflow: Cryofocusing nanomole amounts of CO₂, N₂, and SO₂ from an elemental analyzer for stable isotopic analysis," *Rap. Comm. Mass Spectrom.* **1996**, 10, 953–958.
 52. Galimov, E. M. *The Biological Fractionation of Isotopes*; Academic Press: New York, 1985.
 53. Ghos, Y.; Rutgeerts, P.; van Trappen, G. "¹³CO₂-breath tests in nutritional diagnosis: present applications and future possibilities." In *Clinical Nutrition and Metabolic Research*, Dietz, G., Ed.; Karger: Basel, 1985.
 54. Giesemann, A.; Jager, H.-J.; Norman, A. L.; Krouse, H. R.; Brand, W. A. "On-line sulfur-isotope determination using an elemental analyzer coupled to a mass spectrometer," *Anal. Chem.* **1994**, 66, 2816–2819.
 55. Gohlke, R. S.; McLafferty, F. W. "Early gas chromatography/mass spectrometry," *J. Am. Soc. Mass Spectrom.* **1993**, 4, 367–371.
 56. Gonfiantini, R. "Standards for stable isotope measurements in natural compounds," *Nature* **1978**, 271, 534–536.
 57. Goodman, K. J. "Improvement to a Micromass Optima isotope ratio mass spectrometer to optimize chromatographic resolution," *Anal. Chem.* **1998**, 70,
 58. Goodman, K. J.; Brenna, J. T. "High sensitivity tracer detection using high precision gas chromatography combustion isotope ratio mass spectrometry and highly enriched

- [U-13C]-labeled precursors," *Anal. Chem.* **1992**, *64*, 1088–1095.
59. Goodman, K. J.; Brenna, J. T. "Curve-fitting for resolution of overlapping peaks in high precision gas chromatography-combustion isotope ratio mass spectrometry," *Anal. Chem.* **1994**(a), *66*, 1294–1301.
 60. Goodman, K. J.; Brenna, J. T. "Improved precision by curve-fitting for low signal-to-noise peaks in high precision chromatography-combustion isotope ratio mass spectrometry," *Biolog. Mass Spectrom.* **1994**(b).
 61. Griffiths, H. *Stable Isotopes and the Integration of Biological, Ecological, and Geochemical Processes*; Association of Applied Biologists: Newcastle upon Tyne, UK, 1996.
 62. Hachey, D. L.; Wong, W. W.; Boutton, T. W.; Klein, P. D. "Isotope ratio measurements in nutrition and biomedical research," *Mass Spec. Rev.* **1987**, *6*, 289–328.
 63. Halliday, D.; Rennie, M. J. "The use of stable isotopes for diagnosis and clinical research," *Clin. Sci.* **1982**, *63*, 485–496.
 64. Hayes, J. M. "Practice and principles of isotopic measurements in organic geochemistry." In *Organic Geochemistry of Contemporaneous and Ancient Sediments*, Meinschein, W. G., Ed.; Society of Economic Paleontologists and Mineralogists, Great Lakes Section: Bloomington, Indiana, 1983.
 65. Hobson, K. A.; Sease, J. L.; Merrick, R. L.; Piatt, J. F. "Investigation trophic relationships of pinnipeds in Alaska and Washington using stable ratios of nitrogen and carbon," *Mar. Mam. Sci.* **1997**, *13*, 114–132.
 66. Holt, P. F.; Hughes, B. P. "The preparation of nitrogen samples for mass-spectrographic analyses," *J. Chem. Soc.* **1955**, 95–97.
 67. Horita, J.; Ueda, A.; Mizukami, K.; Takatori, I. "Automatic D and 18O analysis of multi-water samples using H₂ and CO₂ water equilibration methods with common equilibration methods with a common equilibration setup," *Appl. Radiat. Isot.* **1989**, *40*, 801–805.
 68. Ivlev, A. A. "Distribution of carbon isotopes in amino acids of protein fraction of microorganisms as a means of studying the mechanisms of their biosynthesis in the cell," *Biokhimiya (English Translation)* **1986**, *50*, 1605–1615.
 69. Ivlev, A. A. "Distribution of carbon isotopes (13C/12C) in the cell and the temporal organization of cellular processes," *Biophysics* **1991**, *36*, 1078–1087.
 70. Kendall, C.; Coplen, T. B. "Multisample conversion of water to hydrogen by zinc for stable isotope determination," *Anal. Chem.* **1985**, *57*, 1437–1440.
 71. Kendall, C.; Grim, E. "Combustion tube method for measurement of nitrogen isotope ratios using calcium oxide for total removal of carbon dioxide and water," *Anal. Chem.* **1990**, *62*, 526–529.
 72. Kirshenbaum, I. "Physical properties and analysis of heavy water." In *National Nuclear Energy Series*, Urey, H. C.; Murphy, G. M., Eds.; Vol. 4A, McGraw-Hill: New York, 1951.
 73. Kossiakoff, A.; Rice, F. O. "Thermal decomposition of hydrocarbons, resonance stabilization and isomerization of free radicals," *J. Amer. Chem. Soc.* **1943**, *65*, 590–595.
 74. Koziat, J. "Isotope ratio mass spectrometric method for the online determination of oxygen-18 in organic matter," *J. Mass Spectrom.* **1997**, *32*, 103–108.
 75. Levy, R. L. "Trends and advances in design of pyrolysis units for gas chromatography," *J. Gas Chromatogr.* **1967**, *5*, 107–113.
 76. Levy, E. J.; Paul, D. G. "The application of controlled gas phase thermolytic dissociation to the identification of gas chromatographic effluents," *J. Gas Chromatogr.* **1967**, *5*, 136–145.
 77. Lowe, D. C.; Brenninkmeijer, C. A. M.; Tyler, S. C.; Dlugokencky, E. J. "Determination of the isotopic composition of atmospheric methane and its application in the antarctic," *J. Geophys. Res.* **1991**, *96*, 15455–15467.
 78. Markey, S. P.; Abramson, F. P. "Capillary gas chromatography/mass spectrometry with a microwave discharge interface for determination of radioactive-carbon-containing compounds," *Anal. Chem.* **1982**, *54*, 2375–2376.
 79. Marshall, A. G.; Verdun, F. R. "Fourier transforms." In *NMR, Optical, and Mass Spectrometry*; Elsevier: New York, 1990.
 80. Matthews, D. E.; Hayes, J. M. "Isotope-ratio-monitoring gas chromatography-mass spectrometry," *Anal. Chem.* **1978**, *50*, 1465–1473.
 81. McLean, M.; Vestal, M. L.; Teffera, Y.; Abramson, F. P. "Element- and isotope-specific detection for high-performance liquid chromatography using chemical reaction interface mass spectrometry," *J. Chromat.* **1996**, *732*, 189–199.
 82. McLafferty, F. W. "Billionfold data increase from mass spectrometry instrumentation," *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 1–8.
 83. McKinney, C. R.; McCrea, J. M.; Epstein, S.; Allen, H. A.; Urey, H. C. "Improvements in mass spectrometers for the measurement of small differences in isotope abundance ratios," *Rev. Sci. Instrum.* **1950**, *21*, 724–730.
 84. Meier-Augenstein, W. "On-line recording of ¹³C/¹²C ratios and mass spectra in one gas chromatographic analysis," *J. High Res. Chromatogr.* **1995**, *18*, 28–32.
 85. Meier-Augenstein, W.; Brand, W.; Hoffmann, G. F.; Rating, D. "Bridging the information gap between isotope ratio mass spectrometry and conventional mass spectrometry," *Biolog. Mass Spectrom.* **1994**, *23*, 376–378.
 86. Meier-Augenstein, W.; Rating, D.; Hoffmann, G. F.; Wendel, U.; Matthiesen, U.; Schadewaldt, P. "Determination of ¹³C enrichment by conventional and GC-(MS)-C-IRMS," *Isotopes Environ. Health Stud.* **1995**, *31*, 261–266.
 87. Meier-Augenstein, W.; Watt, P. W.; Langhans, C.-D. "Influence of gas chromatographic parameters on measurement of 13C/12C isotope ratios by gas-liquid chromatography-combustion isotope ratio mass spectrometry," *J. Chromat.* **1996**, *A752*, 233–241.
 88. Melzer, E.; Schmidt, H.-L. "Carbon isotope effects on the

- pyruvate dehydrogenase reaction and their importance for relative carbon-13 depletion in lipids," *J. Biol. Chem.* **1987**, *262*, 8159–8164.
89. Merritt, D. A.; Freeman, K. H.; Ricci, M. P.; Studley, S. A.; Hayes, J. M. "Performance and optimization of a combustion interface for isotope ratio monitoring gas chromatography/mass spectrometry," *Anal. Chem.* **1995**, *67*, 2461–2473.
 90. Merritt, D. A.; Hayes, J. M. "Factors controlling precision and accuracy in isotope-ratio monitoring mass spectrometry," *Anal. Chem.* **1994**, *66*, 2336–2347.
 91. Merritt, D. A.; Hayes, J. M. "Nitrogen isotopic analysis by isotope-ratio-monitoring gas chromatography/mass spectrometry," *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 387–397.
 92. Moini, M.; Abramson, F. P. "A moving belt device to couple high-performance liquid chromatography and chemical reaction interface mass spectrometry," *Biol. Mass Spectrom.* **1991**, *20*, 308–312.
 93. Monson, K. D.; Hayes, J. M. "Biosynthetic control of the natural abundance of carbon 13 at specific positions within fatty acids in *Escherichia coli*," *J. Biol. Chem.* **1980**, *255*, 11435–11441.
 94. Monson, K. D.; Hayes, J. M. "Biosynthetic control of the natural abundance of carbon 13 at specific positions within fatty acids in *Saccharomyces cerevisiae*," *J. Biol. Chem.* **1982(a)**, *257*, 5568–5575.
 95. Monson, K. D.; Hayes, J. M. "Carbon isotopic fractionation in the biosynthesis of bacterial fatty acids. Ozonolysis of unsaturated fatty acids as a means of determining the intramolecular distribution of carbon isotopes," *Geochim. Cosmochim. Acta* **1982(b)**, *46*, 139–149.
 96. Morse, A. D.; Morgan, G. H.; Butterworth, A. L.; Wright, I. P.; Pillinger, C. T. "Combined isotopic analysis of nanogram quantities of atmospheric methane," *Rap. Comm. Mass Spectrom.* **1996**, *10*, 1743–1746.
 97. Murphey, B. F. "The temperature variation of the thermal diffusion factors for binary mixtures of hydrogen, deuterium and helium," *Phys. Rev.* **1947**, *72*, 834–837.
 98. Mycke, B.; Hall, K.; Leplat, P. "Carbon isotopic composition of individual hydrocarbons and associated gases evolved from microscaled sealed vessel (MSSV) pyrolysis of high molecular weight organic material," *Org. Geochem.* **1994**, *21*, 787–800.
 99. Newman, A. "The precise world of isotope ratio mass spectrometry," *Anal. Chem.* **1996**, *68*, 373A–377A.
 100. Nier, A. O. "A mass spectrometer for routine isotope abundance measurements," *Rev. Sci. Instrum.* **1940**, *11*, 212–216.
 101. Nier, A. O. "A mass spectrometer for isotope and gas analysis," *Rev. Sci. Instrum.* **1947**, *18*, 398–411.
 102. Nier, A. O.; Gulbransen, E. A. "Variations in the relative abundance of the carbon isotopes," *J. Am. Chem. Soc.* **1939**, *61*, 697–698.
 103. Pella, H.; Colombo, B. *Mikrochim. Acta* **1973**, 697–706.
 104. Prentice, A. M. "Applications of the doubly-labelled-water ($^2\text{H}_2\text{O}$) method in free-living adults," *Proc. Nutr. Soc.* **1988**, *47*, 259–268.
 105. Preston, T.; McMillan, D. C. "Rapid sample throughput for biomedical stable isotope tracer studies," *Biomed. Environ. Mass Spec.* **1988**, *16*, 229–235.
 106. Preston, T.; Owens, N. J. P. "Interfacing and automatic elemental analyser with an isotope ratio mass spectrometer: the potential for fully automated total nitrogen and nitrogen-15 analysis," *Analyst* **1983**, *108*, 971–977.
 107. Preston, T.; Owens, N. J. P. "Preliminary ^{13}C measurements using a gas chromatograph interfaced to an isotope ratio mass spectrometer," *Biomed. Mass Spectrom.* **1985**, *12*, 510–513.
 108. Preston, T.; Slater, C. "Mass spectrometric analysis of stable-isotope-labelled amino acid tracers," *Proc. Nutr. Soc.* **1994**, *53*, 363–372.
 109. Prosser, S. J. "A novel magnetic sector mass spectrometer for isotope ratio determination of light gases," *Int. J. Mass Spectrom. Ion Proc.* **1993**, *125*, 241–266.
 110. Prosser, S. J.; Brookes, S. T.; Linton, A.; Preston, T. "Rapid, automated analysis of ^{13}C and ^{18}O of CO_2 in gas samples by continuous-flow, isotope ratio mass spectrometry," *Biol. Mass Spec.* **1991**, *20*, 724–730.
 111. Prosser, S. J.; Scrimgeour, C. M. "High-precision determination of $^2\text{H}/^1\text{H}$ in H_2 and H_2O by continuous-flow isotope ratio mass spectrometry," *Anal. Chem.* **1995**, *67*, 1992–1997.
 112. Ricci, M. P.; Merritt, D. A.; Freeman, K. H.; Hayes, J. M. "Acquisition and processing of data for isotope-ratio-monitoring mass spectrometry," *Org. Geochem.* **1994**, *21*, 561–571.
 113. Sano, M.; Yotsui, Y.; Abe, H.; Sasaki, S. "A new technique for the detection of metabolites labelled by the isotope ^{13}C using mass fragmentography," *Biomed. Mass Spectrom.* **1976**, *3*, 1–3.
 114. San Pietro, A. *Methods in Enzymology IV*; Academic Press: New York, 1957.
 115. Sansone, F. J.; Popp, B. N.; Rust, T. M. "Stable carbon isotope analysis of low-level methane in water and gas," *Anal. Chem.* **1997**, *69*, 40–44.
 116. Santrock, J.; Studley, S. A.; Hayes, J. M. "Isotopic analyses based on the mass spectrum of carbon dioxide," *Anal. Chem.* **1985**, *57*, 1444–1448.
 117. Schimmelmann, A.; DeNiro, M. J. "Preparation of organic and water hydrogen for stable isotope analysis: Effects due to reaction vessel and zinc reagent," *Anal. Chem.* **1993**, *65*, 789–792.
 118. Schoell, M.; Faber, E. "Carbon and hydrogen isotopic compositions of the NBS 22 and NBS 21 stable isotope reference materials: An inter-laboratory comparison," *Org. Geochem.* **1983**, *5*, 3–6.
 119. Schoell, M.; Hayes, J. M. "Compound-specific isotopic analysis in biogeochemistry and petroleum research," *Org. Geochem.* **1994**, *21*, V.
 120. Schoell, M.; Schouten, S.; Sinninghe-Damste, J. S.; de Leeuw, J. W.; Summons, R. E. "A molecular organic car-

- bon isotope record of miocene climate changes," *Science* **1994**, *263*, 1122–1125.
121. Schoeller, D. A.; Klein, P. D. "A microprocessor controlled mass spectrometer for the fully automated purification and isotopic analysis of breath carbon dioxide," *Bio-med. Mass* **122. Spec.** **1979**, *6*, 350–355.
122. Schoeller, D. A.; Ravussin, E.; Schutz, Y.; Acheson, K. J.; Baertschi, P.; Jequier, E. "Energy expenditure by doubly labeled water: validation in humans and proposed calculation," *Am. J. Phys.* **1986**, *250*, R823–829.
123. Scrimgeour, C. M.; Rollo, M. M.; Mudambo, S. M. K. T.; Handley, L. L.; Prosser, S. J. "A simplified method for deuterium/hydrogen isotope ratio measurements on water samples of biological origin," *Biol. Mass Spec.* **1993**, *22*, 383–387.
124. Sheaff, R. C.; Zhang, Q.; Goodman, K. J.; Guissani, D. A.; Nathanielsz, P. W.; Brenna, J. T. "Linoleate, α -Linolenate and docosahexaenoate recycling into saturated and mono-unsaturated fatty acids is a major pathway in pregnant or lactating adults and fetal or infant rhesus monkeys," *J. Lipid Res.* **1996**, *37*, 2675–2686.
125. Sofer, Z. "Preparation of carbon dioxide for stable carbon isotope analysis of petroleum fractions," *Anal. Chem.* **1980**, *52*, 1389–1391.
126. Sofer, Z. "Chemistry of hydrogen gas preparation by pyrolysis for the measurement of isotope ratios in hydrocarbons," *Anal. Chem.* **1986**, *58*, 2029–2032.
127. Sofer, Z.; Schiefelbein, C. F. "Hydrogen isotope ratio determinations in hydrocarbons using the pyrolysis preparation technique," *Anal. Chem.* **1986**, *58*, 2033–2036.
128. Soni, M.; Frankevich, V.; Nappi, M.; Santini, R. E.; Amy, J. W.; Cooks, R. G. "Broadband Fourier transform quadrupole ion trap mass spectrometry," *Anal. Chem.* **1996**, *68*, 3314–3320.
129. Sprinson, D. B.; Rittenberg, D. "The rate of utilization of ammonia for protein synthesis," *J. Biol. Chem.* **1949**, *180*, 707–712.
130. Svec, H. J. "Mass spectroscopy—ways and means. A historical prospectus," *Int. J. Mass Spectrom.* **1985**, *66*, 3–29.
131. Stark, J. M.; Hart, S. C. "High rates of nitrification and nitrate turnover in undisturbed coniferous forests," *Nature* **1997**, *385*, 61–64.
132. Steyermark, A. *Quantitative Organic Microanalysis*; Academic Press: New York, 1961.
133. Stott, A. W.; Evershed, R. P. " $\delta^{13}\text{C}$ analysis of cholesterol preserved in archaeological bones and teeth," *Anal. Chem.* **1996**, *68*, 4402–4408.
134. Teffera, Y.; Kusmierz, J. J.; Abramson, F. P. "Continuous-flow isotope ratio mass spectrometry using the chemical reaction interface with either gas or liquid chromatographic introduction," *Anal. Chem.* **1996**, *68*, 1888–1894.
135. Tissot, S.; Normand, S.; Guilluy, R., et al. "Use of a new gas chromatograph isotope ratio mass spectrometer to trace exogenous ^{13}C labelled glucose at very low levels of enrichment in man," *Diabetologia* **1990**, *33*, 449–456.
136. Tobias, H. J. "Online compound-specific hydrogen isotopic analysis using continuous-flow isotope ratio mass spectrometry," Ph. D. Thesis, Cornell University: Ithaca, NY, 1997.
137. Tobias, H. J. "Online pyrolysis as a limitless reduction source for continuous flow isotope ratio mass spectrometry of organic hydrogen," *Anal. Chem.* **1997**, *69*, 3148–3152.
138. Tobias, H. J.; Brenna, J. T. "Correction of ion source non-linearities over a wide signal range in continuous-flow isotope ratio mass spectrometry of water-derived hydrogen," *Anal. Chem.* **1996**, *68*, 2281–2286.
139. Tobias, H. J.; Brenna, J. T. "High-precision D/H measurement from organic mixtures by gas chromatography continuous flow isotope ratio mass spectrometry using a palladium filter," *Anal. Chem.* **1996**, *68*, 3002–3007.
140. Tobias, H. J.; Goodman, K. J.; Blacken, C. E.; Brenna, J. T. "Determination of D/H ratios from H_2 and H_2O by continuous flow isotope ratio mass spectrometry," *Anal. Chem.* **1995**, *67*, 2486–2492.
141. Vogler, E. A.; Hayes, J. M. "Carbon isotopic compositions of carboxyl groups of biosynthesized fatty acids." In *Physics and Chemistry of the Earth*, Vol 12, Douglas, A. G.; Maxwell, J. R., Eds.; Pergamon: London, 1980.
142. Walker, J. Q.; Wolf, C. J. "Complete identification of chromatographic effluents using interrupted elution and pyrolysis-gas chromatography," *Anal. Chem.* **1968**, *40*, 711–714.
143. Wada, E.; Tsuji, T.; Sairo, T.; Hattori, A. "A simple procedure for mass spectrometric microanalysis of ^{15}N in particulate organic matter with special reference to ^{15}N -tracer experiments," *Anal. Biochem.* **1977**, *80*, 312–319.
144. Watkins, J. B.; Schoeller, D. A.; Klein, P. D.; Ott, D. G.; Newcomer, A. D.; Hofmann, A. F. " ^{13}C -trioctanoin: a non-radioactive breath test to detect fat malabsorption," *J. Lab. Clin. Med.* **1977**, *90*, 422–430.
145. Wedeking, K. W.; Hayes, J. M. "Exchange of oxygen isotopes between water and organic material," *Isot. Geosci.* **1983**, *1*, 357–370.
146. Weilacher, T. W.; Gleixner, G. G.; Schmidt, H.-L. "Carbon isotope pattern in purine alkaloids a key to isotope discrimination in C_1 compounds," *Phytochemistry* **1996**, *41*, 1073–1077.
147. Werner, R. A.; Kornexl, B. E.; Rosmann, A.; Schmidt, H.-L. "On-line determination of $\delta^{18}\text{O}$ values of organic substances," *Analyt. Chem. Acta* **1996**, *319*, 159–164.
148. Wolfe, R. R. *Radioactive and Stable Isotope Tracers in Biomedicine*; Wiley-Liss, Inc.: New York, 1992.
149. Wong, W. W.; Cabrera, M. P.; Klein, P. D. "Evaluation of a dual mass spectrometer system for rapid simultaneous determination of hydrogen-2/hydrogen-1 and oxygen-18/oxygen-16 ratios in aqueous samples," *Anal. Chem.* **1984**, *56*, 1852–1858.
150. Wong, W. W.; Klein, P. D. "A review of techniques for the preparation of biological samples for mass-spectrometric measurements of hydrogen-2/hydrogen-1 and oxygen-18/oxygen-16 isotope ratios," *Mass Spec. Rev.* **1986**, *5*, 313–342.



Analysis of quantization error in high-precision continuous-flow isotope ratio mass spectrometry

Gavin L. Sacks, Christopher J. Wolyniak, J. Thomas Brenna*

Savage Hall, Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853, USA

Received 10 June 2003; received in revised form 8 August 2003; accepted 12 August 2003

Abstract

High-precision isotope ratio mass spectrometry (IRMS) systems are equipped with digitizers that deliver effective maximum digitization depths of 16 to 24 bits; however, there are no analyses of the proper board depth required to retain high precision in continuous-flow techniques. We report an experimental and theoretical evaluation of quantization error in continuous-flow IRMS (CF-IRMS). CO₂ samples (100 pmol–30 nmol) were injected into a gas chromatography combustion IRMS system (GC-CIRMS). The analog signal was digitized by high precision, 24-bit ADC boards at 10 Hz, and was post-processed to simulate 12, 14, and 16-bit data sets. $\delta^{13}\text{C}_{\text{pdb}}$ values were calculated for all data sets by the conventional “summation” method or by curve-fitting the chromatographic peaks to the exponentially modified Gaussian (EMG) function. Benchmarks of S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) = 0.3, 0.6, and 1.0‰ were considered to assess precision. In the presence of significant quantization noise, curve-fitting required several-fold less CO₂ than the summation method to reach a given benchmark. We derived an equation to describe the theoretical limitations of precision for the summation method as a function of CO₂ admitted to the source and the step size of the boards. Theory was in close agreement with the observed lower limit of precision for the simulated 16-bit data set. Curve-fitting achieved a precision of S.D. <0.3‰ for injections 20-fold smaller than summation for CO₂ samples collected on an IRMS with 16-bit resolution. By mitigating the impact of quantization noise, curve-fitting expands the dynamic range within a single run to include lower analyte levels, and effectively reduces the need for high pumping capacities and high precision ADC boards.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Isotope ratio mass spectrometry; Mass spectrometry; Quantization error; Carbon dioxide

1. Introduction

Isotope ratio mass spectrometry (IRMS) coupled to a gas chromatography–combustion interface (GC–C) can routinely measure relative differences in $^{13}\text{C}/^{12}\text{C}$ isotope ratios to a precision of few parts per million for samples containing 10 ng of sample or less

[1,2]. GC–CIRMS data consists of three concurrent chromatographic traces ($^{44}\text{CO}_2$, $^{45}\text{CO}_2$, $^{46}\text{CO}_2$) from three detectors operated in parallel. Achieving high precision requires careful and consistent definition of background levels and peak integration for all three traces. Most commonly, peak areas are integrated by the “summation” method. The start and end of a peak are detected, and the background is described as a square or trapezoidal area beneath the peak. Raw data are summed over the length of the peak, and the background area is subtracted. Ricci et al. [3] described two

* Corresponding author. Tel.: +1-607-255-9128;
fax: +1-607-255-1033.

E-mail address: jtb4@cornell.edu (J.T. Brenna).

general methods for determining the background using summation; the “individual summation” method, in which the background is defined by connecting low points on either side of the peak, and the “dynamic summation” method, in which low points are connected throughout the chromatogram regardless of the location of peaks.

All IRMS instruments use digitizers to convert analog signal from Faraday cups to digital data, which must be processed to yield isotope ratios. The precision of a digitizer is expressed in terms of bits, where an N -bit board has 2^N steps over a given range. As an example, a 16-bit board has $\sim 65\,000$ steps; if the board has a range of 0–10 V, then the step size of the board is ~ 0.15 mV. The rounding of a continuous signal to discrete steps introduces noise, which is referred to as quantization error or “bit noise”. This effect is shown graphically in Fig. 1, where simulated Gaussian peaks of 24, 16, 14, and 12-bit resolution are presented. At high resolution (24 bits), no quantization noise is noticeable, and the peak appears as a smooth trace. As the resolution decreases, steps become obvious, and the shape of the peak deteriorates. The quality of data reduction in continuous-flow IRMS must depend at least in part on the digitizer depth because the intensity level established for peak start and stop depends on this parameter. As depth decreases, the intensity of

the background is, in general, less well represented by the intensity levels of the peak’s start and stop points. There are no analyses available that establish the relationship between isotope ratio precision and digitization depth.

The effect of digitization depth on precision and accuracy is inextricably linked to data reduction algorithms. The reproducibility of the summation background correction depends in part on the two points that anchor the background line under the peak; imprecision in the measurement of either point multiplies through the entire length of the background segment connecting the points. In the presence of a simple linear background, a background line is easily drawn between any two points on either side of the peak, as shown in Fig. 2a. Chemical noise due to column bleed or contaminant peaks may cause inaccuracy in defining the background, but such noise is usually correlated in all three traces. This covariance may mitigate the effects of chemical noise on the calculated isotope ratio. However, in the case of quantization error, as shown in Fig. 2b, the magnitude and direction of error is uncorrelated among the three traces, and this then poses a special case. Our previous work has shown that peak integration by curve-fitting improves precision and accuracy in cases of low signal-to-noise [4] and overlapping peaks [5]. Background correction

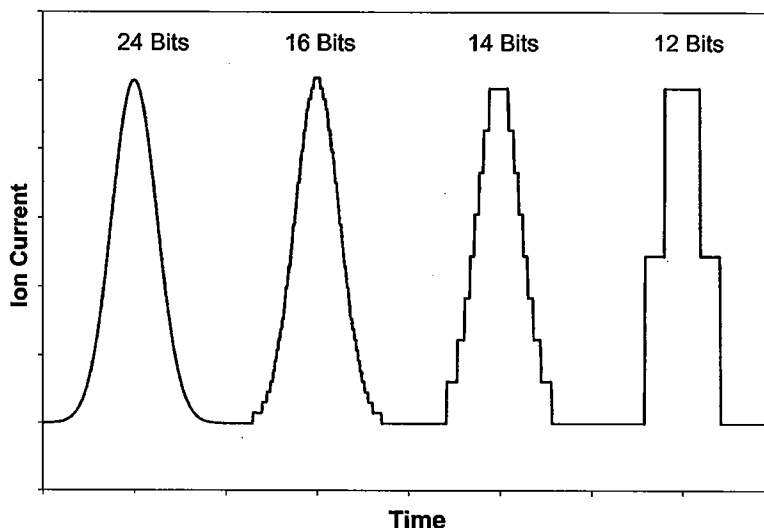


Fig. 1. A simulation of a Gaussian signal collected by ADCs of various resolutions (24, 16, 14, and 12 bits) and quantization errors. At 24-bit resolution, quantization error is not visible, and the peak appears as a smooth trace. At 16 bits, bit noise is evident primarily at the base of the peak. At 12-bit resolution, the signal is barely recognizable as a Gaussian shape.

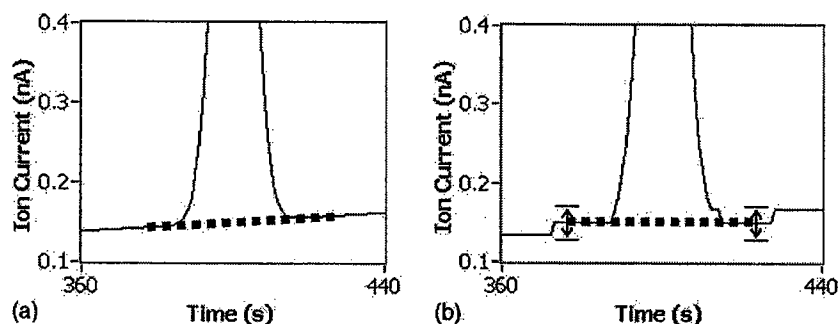


Fig. 2. Simulated chromatographic peaks in the presence of a linearly rising background (a) without and (b) with quantization error. In the presence of quantization error, the true background may fall anywhere within the arrows. Without quantization error background is easily and accurately achieved by connecting points on either side of the peak.

in curve-fitting is not constrained to the actual values represented by the discrete digitization levels, and we hypothesized that it may not be as sensitive to quantization error as summation.

Quantization error is typically not dominant in GC–CIRMS when high precision IRMS data acquisition systems use sufficiently deep digitization boards and signals are sufficiently strong. Noise from other sources, such as chemical noise, is greater than the step size of the digitizers. However, quantization error may become important in two specific situations: (a) in data reduction of minor peaks in a chromatogram where there are fewer steps between baseline and peak top, and (b) when low precision digitizers are used, as is common in low cost IRMS instruments designed primarily for measurements of high abundance samples, such as CO_2 in breath tests. In addition, these systems usually have lower pumping capacity, which limits the flow rate that the IRMS source accepts. The lower inlet flow rates result in smaller signals for equivalent analyte abundance via higher split ratios, making quantization error more prominent. In this report, we evaluate quantization error theoretically and experimentally to determine the limiting the precision achieved by the conventional summation algorithm and by curve-fitting.

2. Experimental

2.1. Instrumentation

A Varian 3400 GC system was coupled via a combustion furnace to one of two gas IRMS instruments:

(a) a FinniganMAT 252 (FMAT252) run in high linearity mode, or (b) an Analytical Precision Products 2003 (APP2003). Both IRMS systems were operated with a source pressure of 1×10^{-6} Torr and had an absolute sensitivity of ~ 5000 mol/ion (1 Torr = 133.322 Pa). The GC–C system is described in detail elsewhere [6]. Briefly, the effluent from the capillary column (60 m \times 0.32 mm, 0.25 μm , BPX70; SGE, Austin, TX, USA) is directed to a combustion furnace filled with CuO and held at 850 $^\circ\text{C}$, and dried in a Nafion water trap before admittance to the IRMS system through an open split. Since CO_2 gas was injected as a sample, the combustion step was not necessary, but was retained in the system to increase the verisimilitude to real GC–CIRMS operating conditions. The FMAT252 has differential pumping and a higher overall pumping capacity, while the APP2003 has only a single turbopump. As a result, the FMAT252 can tolerate higher inlet flow rates. The open split of the FMAT 252 accepted 0.2 ml/min (split ratio = 8.4:1), and the open split of the APP2003 accepted 0.07 ml/min (split ratio = 24:1).

CO_2 (Airgas East, 99.9%) injections were performed by hand consecutively. The split ratio and the injection size were varied to yield between 100 pmol and 30 nmol on column. Four or five replicates were performed for each injection size. The moles of CO_2 in each injection were approximated by assuming ideal gas conditions.

Data was collected on the FMAT 252 using SAXI-CAB [7], a laboratory-built LabVIEW-based [8] data acquisition system employing National Instruments (Austin, TX, USA) 435 \times digitizers yielding

24 bits operating at 10 Hz. Data were collected on the APP2003 using the vendor-supplied 16-bit, 10 Hz data acquisition system. Both systems simultaneously monitored the $m/z = 44$, 45, and 46 cups with >99% duty cycle.

2.2. Data processing

Before data reduction, data collected from the FMAT 252 at 24 bits was rounded on all three traces to simulate 16-, 14-, and 12-bit data sets. The head amplifiers have a maximum signal of 10 V (33 nA for $m/z = 44$), so the step size, Δ , for a given board depth was calculated as:

$$\Delta = \frac{10 \text{ V}}{2^{\text{bits}}} \quad (1)$$

We created simulated data sets by rounding data points to the nearest step:

$$\text{data(quantized)} = \text{round} \left[\frac{\text{data(raw)}}{\Delta} \right] \Delta \quad (2)$$

where, the *round* function rounds the input to the nearest whole number.

The 16-bit data from the APP2003 was used without modification. All data sets were processed using SAXICAB by either the individual summation method or by curve-fitting. The individual summation method used by SAXICAB was adapted from Ricci et al. [3]. Starts and stops of peaks were determined with a slope sensitivity of 0.3 nA/s. The lowest point 2 s before and 2 s after the peak limits were located, and a straight line was drawn between the two points to define the background. In the curve-fitting algorithm, the traces were fit to exponentially modified Gaussian (EMG) functions using the Levenberg–Marquardt algorithm. Mathematical details of the EMG function can be found elsewhere [9].

High-precision isotope ratios are expressed in the delta (‰) notation:

$$\delta^{13}\text{C}_{\text{pdb}} = \frac{{}^{13}R_{\text{spl}} - {}^{13}R_{\text{pdb}}}{{}^{13}R_{\text{pdb}}} \times 1000 \quad (3)$$

where ${}^{13}R_x$ is the ratio of ${}^{13}\text{C}$ to ${}^{12}\text{C}$, SPL refers to the sample, and PDB refers to the international standard, Pee Dee Belemnite, where ${}^{13}R_{\text{pdb}} = 0.0112372$. In our work, $\delta^{13}\text{C}$ of the CO_2 injections were calculated using pulses of standard CO_2 gas that had been indirectly

calibrated to the PDB reference. The contribution of ${}^{17}\text{O}$ to the ${}^{45}\text{CO}_2$ signal was taken into account by the method of Santrock et al. [10]. No outliers were excluded from the reported data.

3. Results and discussion

3.1. Observed effects of quantization error

Fifteen CO_2 injection amounts were used to produce peak areas on the FMAT 252 that varied over 2.5 orders of magnitude. The peaks showed excellent symmetry and narrow peak widths, with a full width at half maximum of <3 s. The mean reproducibility of the area for each injection size, as measured by the area of the $m/z = 44$ signal, was R.S.D. = 13%. Plots of $\delta^{13}\text{C}_{\text{pdb}}$ versus injection size, shown in Fig. 3, are displayed for both the curve-fitting (a) and individual summation (b) methods. The plots are similar in appearance to those presented by others [11] investigating the performance of GC–CIRMS at low signal levels.

In agreement with our previous work [4], we observe modest improvement of precision at low signal levels using the curve-fitting method. Using the summation method, precision deteriorates (S.D. > 1.0‰) for injection sizes less than 400 pmol on column (~50 pmol to the IRMS). Curve-fitting improves this limit to 175 pmol on column (~20 pmol to the IRMS). The integration methods performed comparably and acceptably at large injection sizes. For on-column injections of at least 6.8 nmol (~800 pmol to the IRMS), the individual summation method had a precision of S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) = 0.1‰. The curve-fitting method gave slightly worse precision for large injections, S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) = 0.2‰. It is not obvious why the summation method out-performs the curve-fitting method for very large sample sizes. One possibility is that the precision of the curve-fitting method is limited by differences between the shape of the model EMG function and the shape of real, chromatographic peaks. In this case, increasing the injection size past a certain point would not improve the fit, even though S/N is increasing. Using a different function to describe the peaks may further improve results.

To assess the tolerance of the two integration methods to quantization noise, we evaluated simulated

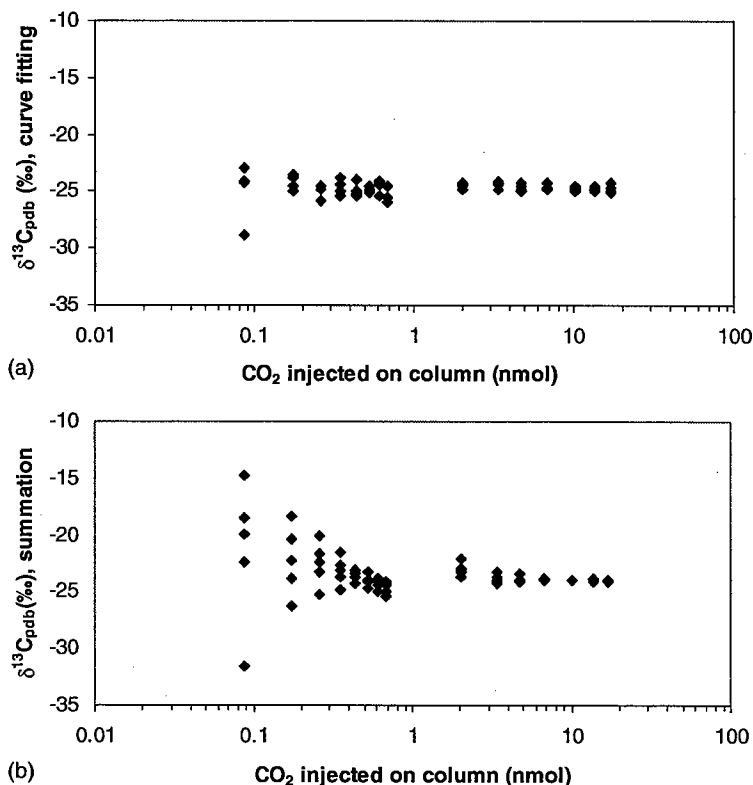


Fig. 3. $\delta^{13}\text{C}_{\text{pdb}}$ vs. CO_2 injected on column for (a) curve-fitting and (b) individual summation algorithms. Data was collected using a homebuilt system at 24 bits from FMAT252.

12, 14, and 16-bit raw data sets generated from the raw 24-bit FMAT252 data, and processed both by curve-fitting and summation. The accuracy of successive injections was very good, even in the presence of bit noise. For each method, the mean $\delta^{13}\text{C}$ for any two injection sizes did not differ significantly. Plots of $\text{S.D.}(\delta^{13}\text{C}_{\text{pdb}})$ versus CO_2 injected on column at all bit resolutions are shown in Fig. 4. Each plot appears to extend asymptotically along the x - and y -axes, and we can evaluate the dependence of precision on quantization error visually; poor performance is indicated by the asymptotic plot moving up and away from the axes. At 24-bit resolution, plots of the summation and curve-fitting methods nearly overlap, except at very small injection amounts, indicating that performance is similar. With increasing quantization error, the minimum amount of CO_2 necessary to reach a given level of precision increases rapidly for the summation method. Curve-fitting is more forgiving; precision from 14- and 16-bit data is comparable to

the 24-bit data. The plot for 12-bit resolution shows some loss of precision, but does not fare as badly as the summation method.

To evaluate the methods objectively, we defined $\text{S.D.}(\delta^{13}\text{C}_{\text{pdb}}) = 0.3, 0.6$ and 1.0‰ as benchmarks for high precision. The data were least-squares fitted to a power function, of the form:

$$\text{S.D.} = A[\text{CO}_2]^B \quad (4)$$

where $[\text{CO}_2]$ is the moles of CO_2 injected on column, S.D. is the observed precision, and A and B are constants. The power function was chosen for empirical reasons, because it modeled the observed data acceptably, and the fitted curves can then be compared. The best-fit lines for both the summation (dashed) and curve-fitting (solid) methods are shown in Fig. 4. From the best-fit equations, we calculated the amount of CO_2 injected on-column necessary to achieve the 0.3, 0.6, and 1.0‰ benchmarks (Fig. 5). With least quantization error (24-bit resolution), the

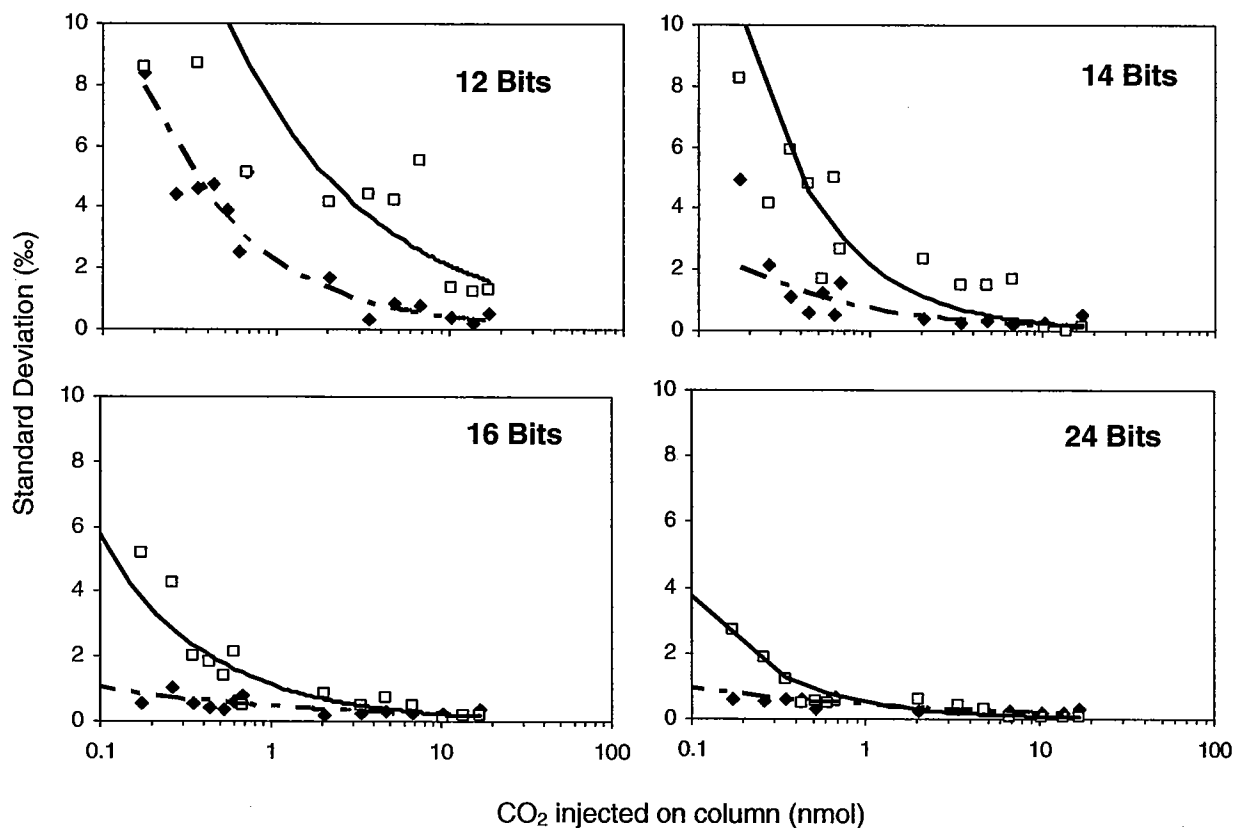


Fig. 4. S.D. ($\delta^{13}C_{pdb}$) vs. CO_2 injected on column at 12-, 14-, 16-, and 24-bit resolutions, calculated by summation (\square) or curve-fitting (\blacklozenge) algorithms. Each point represents four or five replicates. The data for each method and each resolution was fit to a power equation (general form: $S.D. = A[CO_2]^B$, and the best-fit lines are drawn for both methods (solid line: summation; dashed line: curve-fitting).

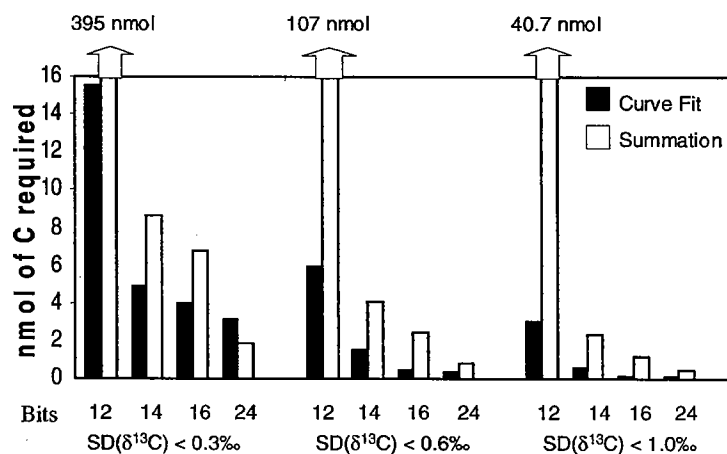


Fig. 5. Carbon required on-column, in nanomoles, to reach a specified level of precision for a given ADC board resolution. Results are shown for data reduced by curve-fitting and summation algorithms.

summation method requires slightly less CO₂ than the curve-fitting method at the 0.3‰ benchmark (1.86 nmol versus 3.19 nmol). At 16-bit resolution, the summation method requires 6.83 nmol, an increase of 267%, compared to a 25% increase for curve-fitting over the same interval. To reach the 0.6‰ benchmark at 16 bits, summation requires an increase of 224% (from 0.77 to 2.50 nmol), compared to 33% for curve-fitting. At 12-bit resolution, the amount of CO₂ on column necessary to achieve S.D. = 0.6‰ by summation is 107 nmol, which far exceeds the capacity of the GC column. Curve-fitting requires only 6 nmol to reach S.D. = 0.6‰ at 12-bit resolution. As was discussed previously, curve-fitting is superior to summation at the 1.0‰ benchmark, even in the absence of quantization noise. At 24 bits, curve-fitting requires 80 pmol to achieve S.D. = 1.0‰, five-fold less than summation; a similar level of improvement in precision is seen at 14- and 16-bit resolution.

To summarize, in the absence of quantization noise, similar amounts of CO₂ are necessary to achieve precision of 0.3–0.6‰ for both integration methods. The summation method requires a dramatic increase in the injection size to maintain this level of precision in the presence of quantization noise, while the curve-fitting method is relatively unaffected. At a lower standard of precision (S.D. = 1.0‰), curve-fitting is superior regardless of the magnitude of quantization error.

3.2. Theoretical limits of quantization error on precision

In IRMS, the signal is recorded as a voltage proportional to the ion current, and can be reported in amperes or in volts. If the signal is reported in volts, the area of the $m/z = 44$ signal, A_{44} , is related to the moles of ⁴⁴CO₂ that enters the IRMS, [⁴⁴CO₂], by the equation:

$$A_{44} = [^{44}\text{CO}_2] \frac{N_a e}{E} R_\Omega \quad (5)$$

where N_a is Avogadro's number, e is the fundamental charge, E is the absolute sensitivity of the IRMS in molecules/ion, and R_Ω is the feedback resistance of the amplifier.

In the summation method, the background is defined by drawing a line between two background points, (t_1, y_1) and (t_2, y_2); the background area, A , is the

trapezoidal region between this line and the time axis. We can calculate this area by the equation:

$$A(\text{background}) = \frac{1}{2} W(y_1 + y_2), \quad (6)$$

where $W = t_2 - t_1$

Quantization noise is uniformly distributed over an interval and the error for a single measurement is:

$$\sigma_y = \frac{\Delta}{\sqrt{12}} \quad (7)$$

where Δ is the minimum step size of the acquisition boards. A full derivation of this can be found in Haykin's text on digital communication [12]. Assuming that quantization error at y_1 and y_2 is uncorrelated, we can use standard techniques for propagation of errors to determine the total quantization error in measuring the background area, σ_A :

$$\sigma_A = \frac{W\Delta}{2\sqrt{6}} \quad (8)$$

It has been noted that evaluating the effect of chemical noise on precision of isotope ratios is difficult, because this noise is usually highly correlated between the major and minor traces [11]. Unlike chemical noise, quantization noise on each trace should be uncorrelated. This greatly simplifies calculation of the propagation of errors for the relation of the observed isotope ratio, ⁴⁵ R_{obs} , to the actual isotope ratio, ⁴⁵ R_{act} :

$$^{45}R_{\text{obs}} = \frac{A_{45} \pm \sigma_{45}}{A_{44} \pm \sigma_{44}} = ^{45}R_{\text{act}} \pm \sigma_{\text{obs}} \quad (9)$$

where

$$\sigma_{\text{obs}} = ^{45}R_{\text{act}} \sqrt{\left(\frac{\sigma_{45}}{A_{45}}\right)^2 + \left(\frac{\sigma_{44}}{A_{44}}\right)^2} \quad (10)$$

The standard error can be rearranged and expressed in terms of parts per thousand:

$$\begin{aligned} \sigma_{\text{ppt}} &= 1000 \times \frac{\sigma_{\text{obs}}}{^{45}R_{\text{act}}} \\ &= 1000 \times \sqrt{\left(\frac{\sigma_{45}}{A_{45}}\right)^2 + \left(\frac{\sigma_{44}}{A_{44}}\right)^2} \end{aligned} \quad (11)$$

At natural abundance, $A_{45} \sim 0.011A_{44}$. Assuming the feedback resistance is 100× larger for the 45 cup than for the 44 cup, $\Delta_{45} = 0.01\Delta_{44}$. Combining this and Eqs. (5), (8) and (11), we arrive at:

$$\sigma_{\text{ppt}} = \frac{276W\Delta_{44}E}{[^{44}\text{CO}_2]N_a e R_\Omega} \quad (12)$$

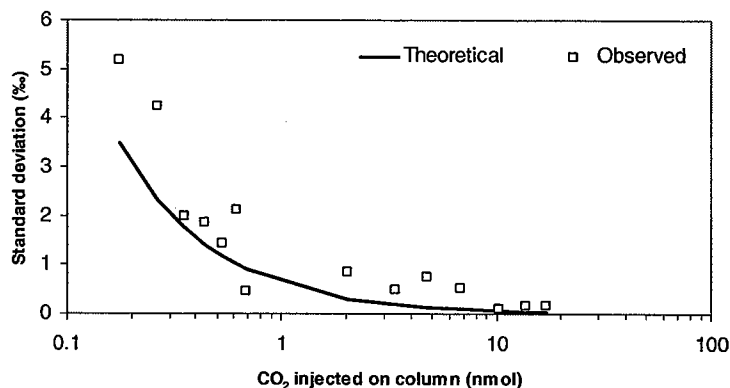


Fig. 6. S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) vs. CO_2 injected on column for simulated 16-bit data. The line indicates the theoretical limit of precision as a function of injection size, as calculated from Eq. (12).

At natural abundance, a standard deviation of $\sigma_{\text{ppt}} = 1.0$ is approximately equivalent to $\text{S.D.}(\delta^{13}\text{C}_{\text{pdb}}) = 1.0\%$. We used Eq. (12) to predict the standard deviation as a function of injection size at 16-bit resolution, compensating for an open split ratio of 8.4:1. The integration window, W , was assumed to be constant at 10 s, $E = 5000$, and $R_{\Omega} = 3 \times 10^8 \Omega$. A plot of the calculated limits compared to the observed precision at 16 bits is shown in Fig. 6. There is good agreement between theory and experiment. The calculated precision is within a factor of five of the observed precision for all injection sizes. More striking, the calculated precision is a “lower limit”, as nearly all the measured precisions lie above the theoretical prediction. The biggest discrepancies occur for large injection sizes, where the effect of quantization error is minimized, and other sources of error (e.g. contaminants) may dominate.

Eq. (12) can also be used to demonstrate that quantization error should be negligible for signals acquired with 24-bit digitizers. Eq. (12) predicts that only 0.6 pmol of CO_2 to the IRMS should be necessary to achieve a precision of 0.5% if quantization error is the only limiting factor. However, counting statistics dictate that a minimum number of ions must be formed to achieve a specified precision to overcome the shot-noise limit. Merritt and Hayes [11] give this equation as:

$$\sigma_{\delta}^2 = \frac{(2 \times 10^6)(1 + R)^2}{EmN_a R} \quad (13)$$

where σ_{δ} is the shot noise limited standard deviation, R is the natural abundance isotope ratio, E is the ionization efficiency, m is the moles of CO_2 , and N_a is Avogadro's number. Substituting $E = 5000$ and $R = 0.011$, we find that 6 pmol of analyte is required to achieve $\text{S.D.} < 0.5\%$. Therefore, when high precision 24-bit boards are used, the effect of quantization error is superseded by shot noise.

Our theoretical treatment gives insight into why curve-fitting is less sensitive to quantization error than summation. In the summation methods we have discussed, imprecision in a single data point chosen as the background is multiplied throughout the background correction; for a peak width of N data points, the total quantization error scales as N . In contrast, the algorithms used in curve-fitting minimize the sum of squares between the fit curve and every data point. In curve-fitting, the quantization noise for each individual point is averaged over the entire curve; for N data points, the total quantization error scales as $N^{1/2}$. For a peak width of 10 s and a sampling rate of 10 Hz, this translates into a 10-fold reduction of quantization error.

The theoretical treatment we describe is appropriate for understanding the effects of quantization error on summation integration methods that choose single points on either side of the peak to define a background. It does not examine the limits of other classes of data reduction methods. An obvious improvement to the summation integration method would be to average n points on either side of the peak, which would increase the effective number of bits of

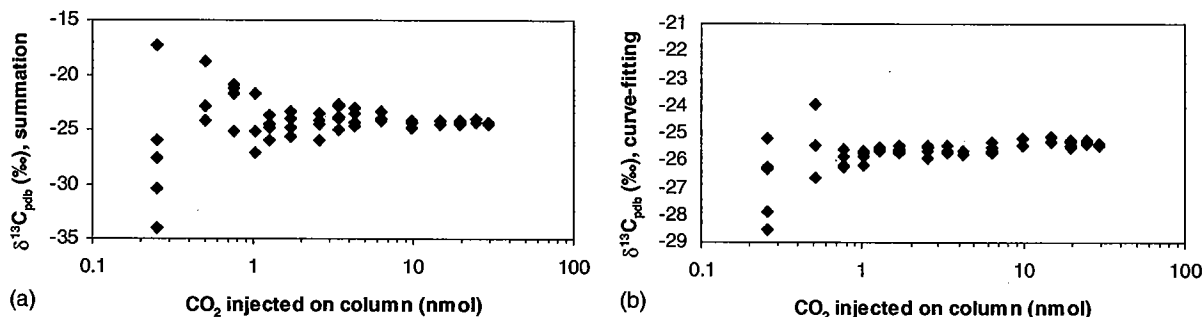


Fig. 7. $\delta^{13}\text{C}_{\text{pdb}}$ vs. CO_2 injected on column for (a) individual summation and (b) curve-fitting algorithms. Data was collected at 16 bits and 10 Hz on the APP2003.

the background measurement by $n^{1/2}$. While this approach could work well for isothermal runs with constant background, it is much less suitable for complex GC–CIRMS chromatograms, where it is not obvious *which* points should be averaged; that is, which points represent pure background and do not contain chemical noise or the tail ends of peaks. Ricci et al., observed that the averaging method gives slightly higher background values than other corrections [3]. They also reported that the dynamic background correction (which uses single points) yielded improved δ -values over the averaging method. Thus, a method may be insensitive to quantization error, but may still give worse results due to other variables.

3.3. Improving precision on a 16-bit IRMS

To test the effectiveness of curve-fitting on GC–CIRMS data acquired by low precision digitiz-

ers, we ran multiple CO_2 injections on an APP2003 using 16-bit digitizers, and otherwise in similar fashion to the work on the FMAT252. A plot of $\delta^{13}\text{C}_{\text{pdb}}$ versus injection size is shown for summation (Fig. 7a) and curve-fitting (Fig. 7b). Fig. 8 shows a plot of S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) as a function of injection size for both integration methods. The theoretical limit on the summation method, calculated from Eq. (12), is shown in the same figure as a dashed line. The observed precision for the summation method agrees well with theoretical predictions; most of the data points lie just above the lower limit curve. Almost 15 nmol of CO_2 on column are necessary to achieve a precision of $<0.3\%$ using the summation method. Using curve-fitting, only 0.76 nmol are necessary to reach that level of precision, a 20-fold improvement. This is greater than the two-fold advantage seen by curve-fitting the 16-bit data from the FMAT 252. One possible explanation is that the APP2003 data is

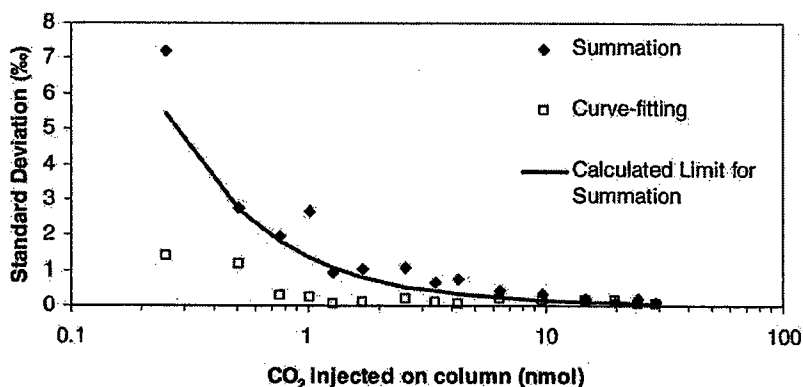


Fig. 8. S.D. ($\delta^{13}\text{C}_{\text{pdb}}$) vs. CO_2 injected on column for runs on APP2003 with 16-bit boards. The dashed line indicates the theoretical limit of quantization error on precision as a function of injection size, as calculated from Eq. (12).

affected primarily by bit noise, while the FMAT252 data has other sources of noise that cannot be eliminated by curve-fitting. The FMAT252 accepts a three-fold higher flow rate than the APP2003, so there is likely more chemical noise in the FMAT252 signal.

The relative immunity to quantization error with curve-fitting permits the IRMS to be run at lower inlet flow rates, which effectively increases quantization noise by decreasing the number of steps between background and peak with relatively little influence on chemical noise. The advantages of lower inlet flow rates are a longer lifetime for the filament, and reduced need for pumps and pumping capacity. These benefits, plus the reduced need for expensive ADC boards, should make high-precision GC–CIRMS more amenable to portable and low-cost applications. In principle, statistical considerations define the lower limits of flow rates. However, counting statistics dictate that S.D. = 0.5‰ requires 6 pmol of CO₂ to the source for a typical continuous flow IRMS ($E = 5000$), and GC–CIRMS applications usually work well above this limit. Thus, modestly lower resolution and inlet flow rates should not significantly affect performance, so long as appropriate integration techniques are used.

4. Conclusions

Data reduction using curve-fitting is more robust than the conventional summation method in the presence of even modest levels of quantization error. Using data obtained on high precision digitizers, the curve-fitting algorithm required several-fold less CO₂ to reach benchmarks of high precision (S.D. = 0.3, 0.6, and 1.0‰) at any of the three simulated board depths (12, 14, or 16 bits). The poor performance of the summation algorithm was particularly noticeable at the 12-bit resolution, where S.D. < 1.0‰ could not be reached even at the maximum injection size allowed by the dynamic range of the Faraday cups. We

have derived an expression that describes the influence of quantization noise on isotope ratios calculated from raw IRMS data, and shown that it accurately predicts the lower limit of precision. Our theoretical treatment assumes that quantization error is uncorrelated between the $m/z = 44$ and 45 signals, and is appropriate for any data reduction algorithm that uses single points on either side of the peak to describe the background.

Curve-fitting substantially improved precision on GC–CIRMS data collected by an instrument with 16-bit digitizers. The summation algorithm required 15 nmol of CO₂ on-column to achieve a precision of S.D. = 0.3‰, while curve-fitting required only 0.76 nmol. Thus, IRMS with 16-bit ADC boards achieved high precision for less than 1 nmol of C on column, a common benchmark for GC–CIRMS applications, despite using 16-bit ADC boards. Lower inlet flow rates, enabling reduced pumping requirements, may be an important advantage in some applications.

References

- [1] W. Meier-Augenstein, *J. Chromatogr. A* 842 (1999) 351.
- [2] J.T. Brenna, T.N. Corso, H.J. Tobias, R.J. Caimi, *Mass Spectrom. Rev.* 16 (1997) 382.
- [3] M.P. Ricci, D.A. Merritt, K.H. Freeman, J.M. Hayes, *Org. Geochem.* 21 (1994) 561.
- [4] K.J. Goodman, J.T. Brenna, *J. Chromatogr. A* 689 (1995) 63.
- [5] K.J. Goodman, J.T. Brenna, *Anal. Chem.* 66 (1994) 1294.
- [6] K.J. Goodman, J.T. Brenna, *Anal. Chem.* 64 (1992) 1088.
- [7] G.L. Sacks, J.T. Brenna, J.T. Sepp, in: *Proceedings of the 49th ASMS Conference on Mass Spectrometry*, Chicago, IL, 2001.
- [8] National Instruments, Austin, TX, 2000.
- [9] N. Dyson, *Chromatographic Integration Methods*, Royal Society of Chemistry, Cambridge, UK, 1990.
- [10] J. Santrock, S.A. Studley, J.M. Hayes, *Anal. Chem.* 57 (1985) 1444.
- [11] D.A. Merritt, J.M. Hayes, *Anal. Chem.* 66 (1994) 2336.
- [12] S. Haykin, *An Introduction to Analog and Digital Communications*, Wiley, New York, 1989.



Pergamon

PII: S0146-6380(98)00157-0

Org. Geochem. Vol. 29, No. 5-7, pp. 1101-1117, 1998
© 1998 Elsevier Science Ltd. All rights reserved.
Printed in Great Britain
0146-6380/98 \$ - see front matter

Analytical improvements in irm-GC/MS analyses: Advanced techniques in tube furnace design and sample preparation

LEROY ELLIS* and ANN L. FINCANNON

ARCO Exploration and Production Technology, 2300 W. Plano Parkway, Plano, TX 75075, U.S.A.

Abstract—Peak resolution is an essential requirement for achieving accurate irm-GC/MS compound-specific isotope measurements. Obtaining accurate isotope data from petroleum extracts, in particular, has proven especially difficult due to co-elutions that typically comprise crude oil mixtures. New enhancements in peak resolution in irm-GC/MS analyses have been achieved by substituting a polyimide-coated glass capillary as the furnace reactor. The advantages of the design are significant, and include: (1) incorporation of a zero dead-volume combustion reactor, resulting in almost no loss in gas chromatographic peak resolution, equating to less compound interferences and more accurately measured isotope values and (2) use of direct capillary-to-capillary leak tight, zero dead-volume connections that reduce the incidence of leak problems generally encountered as a result of using fittings of substantially different sizes. In addition, improvements in analytical chromatography using size-exclusion techniques such as urea adduction and molecular sieving reduces the number of components in a sample, and have resulted in the acquisition of more accurate isotope data. Experiments comparing paraffin isotope measurements for whole oil samples to those of urea adducted samples of the same oil have shown that differences of 2‰ are not uncommon. Data from experiments with isotope standards indicate that urea adduction and molecular sieving techniques have no observable fractionation effect on the measurement of isotope values for paraffin components in these mixtures. Considering the intensive effort and expense in generating irm-GC/MS data, the use of relatively inexpensive chromatography techniques such as urea adduction and molecular sieving in isolating components of interest for accurate isotope analysis is justified. © 1998 Elsevier Science Ltd. All rights reserved.

Key words—Isotope, irm-GC/MS, combustion, petroleum, urea adduction, molecular sieving

INTRODUCTION

Isotope geoscience has established itself firmly as an integral component of applied research, development and resource exploration throughout the world. Information derived from isotope measurements of both inorganic and organic sample components offers unique insights into dynamic geologic and biogeochemical processes occurring in past and present day terrestrial, aquatic, atmospheric and subsurface environments. Particularly for petroleum geoscience, the advent of high resolution gas chromatographic techniques has opened the way for detailed analysis of individual components in complex hydrocarbon mixtures (Sofer *et al.*, 1991). Development of high resolution gas chromatography instrumentation used in tandem with isotope ratio mass spectrometers now permits the accurate measurement of carbon, oxygen, sulfur and nitrogen isotopes on individual sample components (Matthews and Hayes, 1978; Hayes *et al.*, 1990; Brenna, 1994; Merritt and Hayes, 1994; Wong *et al.*, 1995). Future analytical refinements of isotope ratio monitoring-gas chromatography mass spectrometry (irm-GC/MS) are directed towards

further improvements in compound separation and analysis on chromatographic columns used in gas chromatographs, and also advanced sample preparation techniques (Meier-Augenstein, 1997).

Even with the significant advances in compound separation and resolution made possible by the application of high-resolution gas chromatography, it is not expected that complete separation of complex mixtures comprising hundreds to thousands of compounds will be achieved in the short term. While in general this may not even be viewed as necessary for routine GC-MS applications, it is of critical importance in relation to obtaining accurate isotope values in irm-GC/MS applications (Meier-Augenstein, 1997). A minor component co-eluting with a compound of interest can have a significant effect on the measured isotope value if their respective carbon isotopes are significantly different (Ricci *et al.*, 1994; Merritt *et al.*, 1994). Application of mathematical deconvolution techniques in data processing by Goodman and Brenna (1994) have successfully enabled accurate isotope determinations on closely eluting chromatographic peaks with valley overlaps as large as 40%, however significant co-elutions in complex mixtures are less likely to be 'isotopically' deconvoluted with any confidence. Incorrect isotope values achieve very little in re-

*To whom correspondence should be addressed.

lation to data interpretations as variability of only 1–2% is enough to alter and/or invalidate outcomes for many petroleum geochemical applications.

Obtaining simpler, less complex sample fractions through the use of chromatography is, therefore, desired. In measuring *n*-alkane profiles, presently a common application of *irm*-GC/MS petroleum applications, the use of urea adduction and molecular sieve chromatography techniques have proven to be beneficial. Application of either of these techniques results in the adduction and isolation of paraffin fractions containing only *n*-alkanes, monomethyl, one-ring cyclic and in some cases dimethyl alkyl paraffins (Murphy, 1969; Rubinstein and Strausz, 1979; Hoering and Freeman, 1984; Ellis, 1989; West *et al.*, 1990). These simplified paraffin fractions allow for improved accuracy in isotope measurements without interference from co-eluting components or unresolved complex mixtures (UCM) such as those comprising the background matrix.

In this paper, we report on the improvements in component peak resolution obtained from introduction and application of a capillary furnace reactor* in place of conventional tube furnaces (Ellis and Fincannon, 1997). Improvements in isotope data accuracy derived from enhanced peak resolution are also shown in relation to separation of petroleum condensates and a crude oil example. The application of analytical size-exclusion techniques including urea adduction and molecular sieving with respect to potential isotope fractionation issues are also addressed.

EXPERIMENTAL

Samples

The "VGmix" represents a four component mixture of *n*-C₁₀, *n*-C₁₁, *n*-C₁₂ and methyl decanoate reference compounds (ISOCHROM 1-Isotope ratio mass spectrometer user manual, VG Isotech, December 1989). The crude oil saturate fraction used in Fig. 5 is an example of an Indonesian petroleum (95X3797). The condensate used in Fig. 6 to illustrate resolution of gasoline range components was obtained from the North Slope, Alaska (86X1802). The light crude oil (96X4289) used in the sample preparation adduction work represented in Fig. 8 is a petroleum (36.7-API), obtained from Indonesia. The paraffin isotope standard mixture used to test potential fractionation effects of urea adduction sample preparation comprised a synthetic mixture of C₁₂–C₃₀ *n*-alkanes.

*Note added in proof. Since this paper was accepted for publication, a report has been made of the use of a capillary furnace tube for *irm*-GC/MS applications, cf. Goodman K. J. (1998) *Analytical Chemistry* 70: 833–837.

Urea adduction and molecular sieving

The paraffin isotope standard mixture and the whole oil saturate fraction (96X4289) were urea adducted at Baseline Resolution (Plano, TX) to isolate the paraffins (Murphy, 1969). In brief, urea adduction involves application of a saturated solution of urea in methanol to an isolated petroleum saturate fraction dissolved in a toluene-methanol solvent mixture. The urea mixture is allowed to stand overnight for complete adduction, after which, the solvent is pipetted from the urea precipitate. With small sample loadings, a solvent rinse is used to ensure complete recovery of the non-adducted fraction. The urea clathrate in the form of crystals is dried under nitrogen, then the adducted *n*-alkanes, monomethyl alkanes and monocyclic alkanes are recovered with the addition of pentane or hexane after dissolution of the clathrate in water. Similarly, the nonadducted branched/cyclic saturate fraction is also recovered by drying the methanol/toluene solvent under nitrogen, dissolving any residual precipitated urea in water and then adding pentane or hexane.

Molecular sieving analyses were performed at the Houston Advanced Research Laboratories (HARC, Houston, TX). The proprietary HARC technique, referred to as the "PINS" procedure, separates paraffins, monomethyl and monocyclic alkanes and isoprenoids from other saturate compounds (Nolte, 1991). Molecular sieving was also performed at the Australian Geological Survey Organisation (Canberra, ACT) using column chromatography techniques and silicahite molecular sieves.

irm-GC/MS analyses

A Hewlett Packard 5890 gas chromatograph equipped with a model 7673 automatic cool on-column injector system is used in tandem with a Model SIRA II (MicroMass, U.K.) isotope ratio monitoring mass spectrometer. A fused silica column of 60 m × 0.25 mm ID coated with a DB-1 stationary phase (J&W Scientific) was used for analyses of the *n*-alkane standards. Helium was used as carrier gas at a linear gas velocity of 33 cm s⁻¹. For VGmix runs, the GC oven was programmed from 100°C (5 min hold time) to 180°C at 5°C min⁻¹. For condensate runs the GC oven was programmed from 30°C (5 min hold time) to 30°C at 3°C min⁻¹, then from 30 to 310°C (20 min hold time) at 10°C min⁻¹. For saturate fraction runs, the GC oven was programmed from 100°C (0 min hold time) to 310°C at 5°C min⁻¹ (9 min hold time). The combustion furnace was maintained at 900°C during all analyses. Typical MS operating conditions were: ionization energy 100 eV, trap current 600 μA, accelerating voltage 2550 kV. CSIA analyses on the whole oil saturate, molecular sieve and urea adduct fraction reported in Table 4 were

Capillary C



Gas Chrom

This area

performed at the Organisation.

RESUL

irm-GC/MS system

Typical *irm*-GC/MS system of organic is a polyimide-coated chromatograph, a referred to as a "fi an open split, an and a data proce sample of a part injected into the ponents then inte terial and are ch the same time, the proximately 300°C various compone their respective b are vaporized, the rier gas from the tion reactor or f approximately 90 and in the preser oxide catalyst (e.g these volatile org metal oxide and dioxide and wau metal oxide has "bound-oxygen", "regenerated" ei vation process (2 tion of the sar elution of sampl contamination o Water is remove water separator

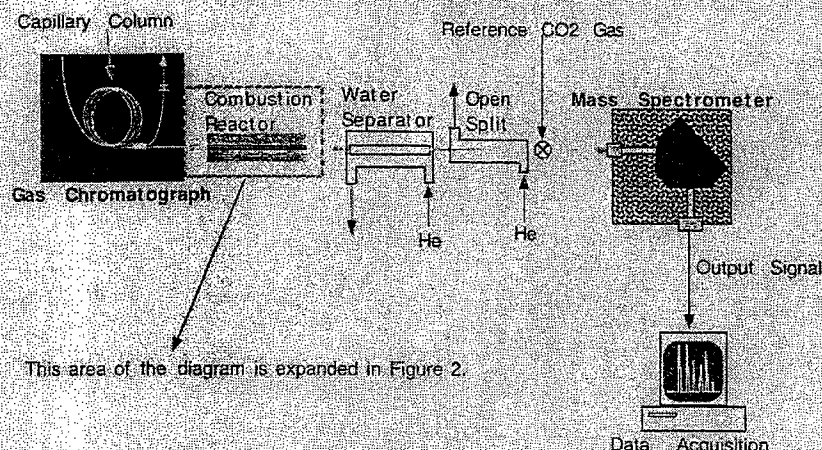


Fig. 1. irm-GC/MS system overview.

performed at the Australian Geological Survey Organisation.

RESULTS AND DISCUSSION

irm-GC/MS system overview

Typical irm-GC/MS systems used in the measurement of organic isotopes are generally comprised of a polyimide-coated glass capillary column in a gas chromatograph, a combustion reactor (sometimes referred to as a "furnace"), a water removal system, an open split, an isotope ratio mass spectrometer and a data processing unit (Fig. 1). In brief, a sample of a particular GC amenable mixture is injected into the column, where the sample components then interact with the column phase material and are chromatographically separated. At the same time, the column is also heated (e.g. to approximately 300°C) to "distill" and volatilize the various components of the mixture according to their respective boiling points. As the components are vaporized, they are transported via helium carrier gas from the capillary column into the combustion reactor or furnace, where the temperature is approximately 900°C. At this elevated temperature and in the presence of a single combination metal oxide catalyst (e.g. copper, nickel and/or platinum), these volatile organic components "react" with the metal oxide and are converted/oxidized to carbon dioxide and water combustion products. Since the metal oxide has only a limited amount of available "bound-oxygen", the catalyst needs to be frequently "regenerated" either *in situ* or as a separate activation process (Merritt *et al.*, 1995). Initial redirection of the sample flow to a vent/FID during elution of sample solvent is performed to eliminate contamination/overloading of the furnace oxidant. Water is removed from the sample stream via the water separator (Merritt *et al.*, 1995). Excess carbon

dioxide formed during combustion, which might otherwise overload the detector electronics of the mass spectrometer is removed through an open split prior to sample entry into the mass spectrometer. The open split maintains a constant pressure, thereby removing any pressure fluctuations that could damage the source electronics. The mass spectrometer subsequently detects and measures separately each respective carbon dioxide isotope as that gas flows through and generates a representative signal. The respective signals are then processed to produce a chromatographic trace illustrating a curve with "peaks" which are representative of the respective carbon dioxide isotopes in the sample. These measurements are further processed to generate numerical data (e.g. the area under the peaks are integrated) which, in turn, are then interpreted in accordance with known relationships to standards in order to arrive at an "isotope measurement" representative of the sample component.

For reliable isotope values, peak integrity is required as the sample components are transported via the carrier gas through to the mass spectrometer. Unfortunately, the need for a combustion furnace, a water removal system and an open split in irm-GC/MS systems introduce a myriad of potential problems related to an increased number of tubing connections. This is especially true for the furnace interface, which typically uses a larger bore glass or ceramic furnace tube connected to capillary lines. Besides the obvious "leak" problems associated with connecting tubing of different internal and external dimensions, all these problems have the added potential of suffering from "dead-volume" and peak separation and resolution is often severely compromised as a result. Dead-volume can be defined as any point in the carrier gas flowpath where the volume in the path increases, e.g. at tubing connections along the flow-

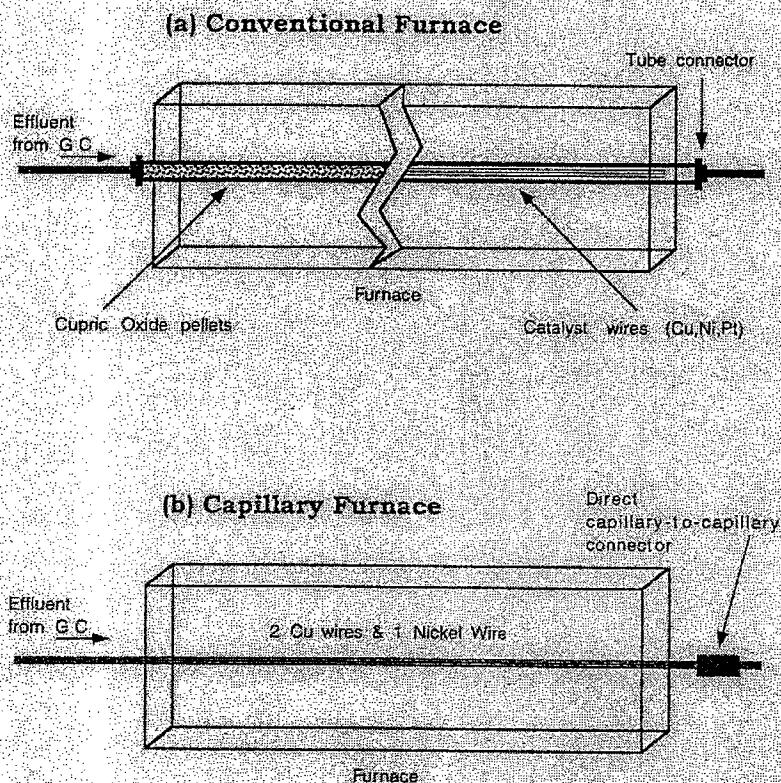


Fig. 2. Comparison of combustion interfaces comparing commercially available glass and ceramic furnace designs with that of the capillary furnace design.

path, etc. As the sample encounters dead-volume along the flowpath, the back pressure on the sample decreases and flow slows as the carrier gas and sample expand and dissipate into the additional space available. With irm-GC/MS systems of the type used to measure hydrocarbon isotopes, dead-volume can typically present problems in the carrier gas flowpath as it passes into the combustion reactor or furnace from the GC.

Tube furnace design

Furnaces used in irm-GC/MS systems contain a metal oxide catalyst (copper, nickel and/or platinum) in pelletized or extruded wire form, and generally use a larger diameter glass or ceramic tube (e.g. 0.6 mm ID) to form the sample flowpath [Fig. 2(a)]. This relatively large diameter tube is connected directly to the smaller capillary GC column (e.g. approximately 0.25 mm ID). The connection of the larger tube in the furnace to the capillary tube of the GC column can often result in the creation of significant dead-volumes. Dead-volumes have the potential to severely affect the final results of the carbon dioxide isotope measurements, and can create broader peaks with "peak

tailoring" on the chromatographic trace. Maintaining leak-free connections with this type of system furnace configuration is problematic. Accordingly, improvements are needed in the sample flowpath to eliminate dead-volume, and thereby maintain peak integrity of sample components after they leave the chromatographic column.

Replacement of standard glass or ceramic tube furnaces with a capillary combustion tube furnace [Fig. 2(b)], has been shown to be extremely effective in maintaining peak resolution through a smaller and more leak resistant system. Any dead-volume attributable to the increased diameter of the previous furnace tube designs is also substantially eliminated. Minimizing peak broadening effects caused by perturbed gas flow through larger internal volume furnace reactors was achieved by substituting a polyimide-coated fused silica capillary as the furnace reactor. Preferably, a length of deactivated polyimide-coated fused silica capillary tube having basically the same ID as that of the GC capillary column (between 0.22 and 0.33 mm) is used in forming the combustion tube. In addition, as the polyimide-coating of the capillary column is unstable at the elevated temperatures (i.e. 900°C)

(a)

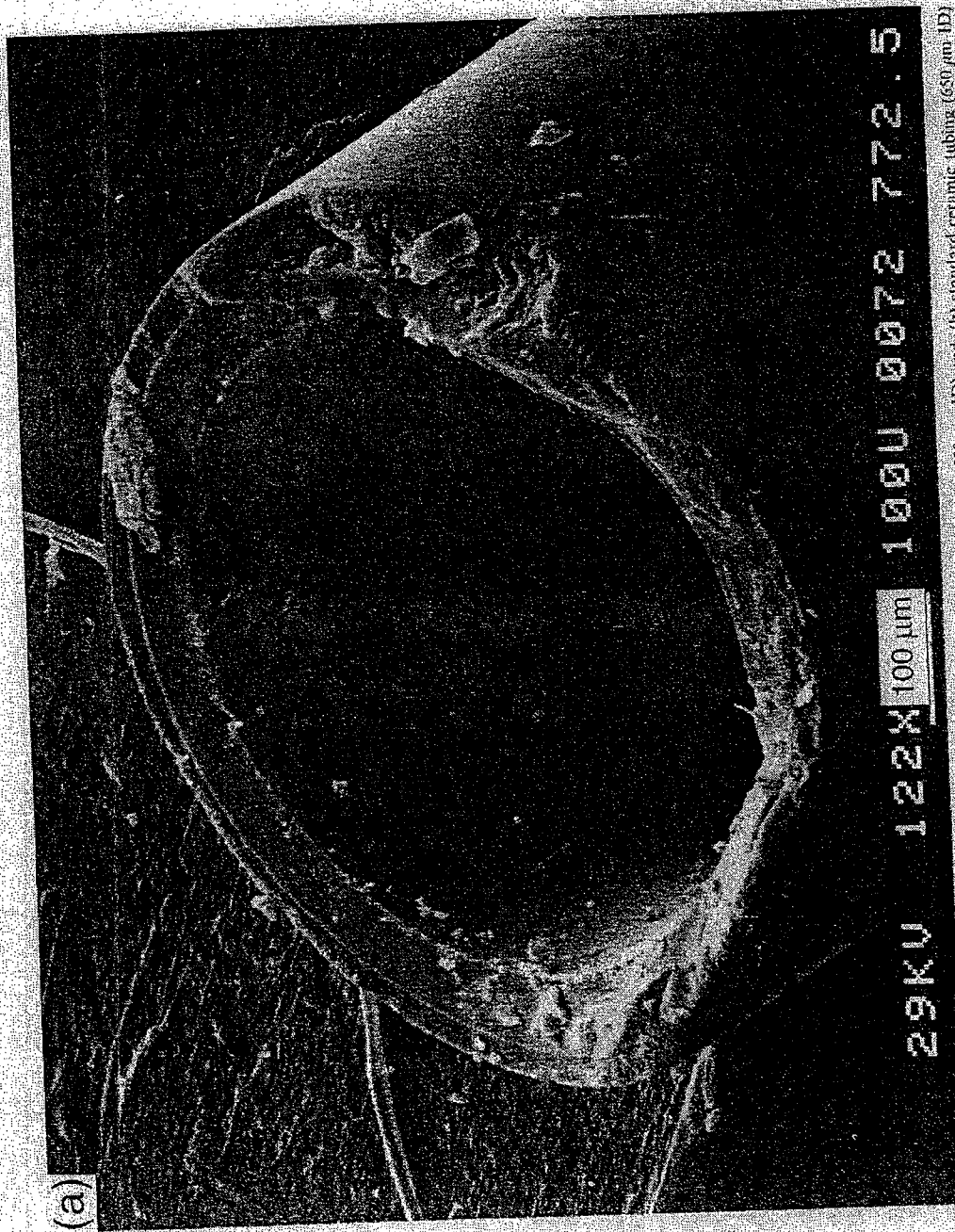


Fig. 3. Scanning electron microscope images showing comparison of (a) capillary fused silica tubing (530 µm ID) and (b) standard ceramic tubing (650 µm ID) used for imm-GC/MS furnace tubes.



Fig. 3 (continued)

Response →

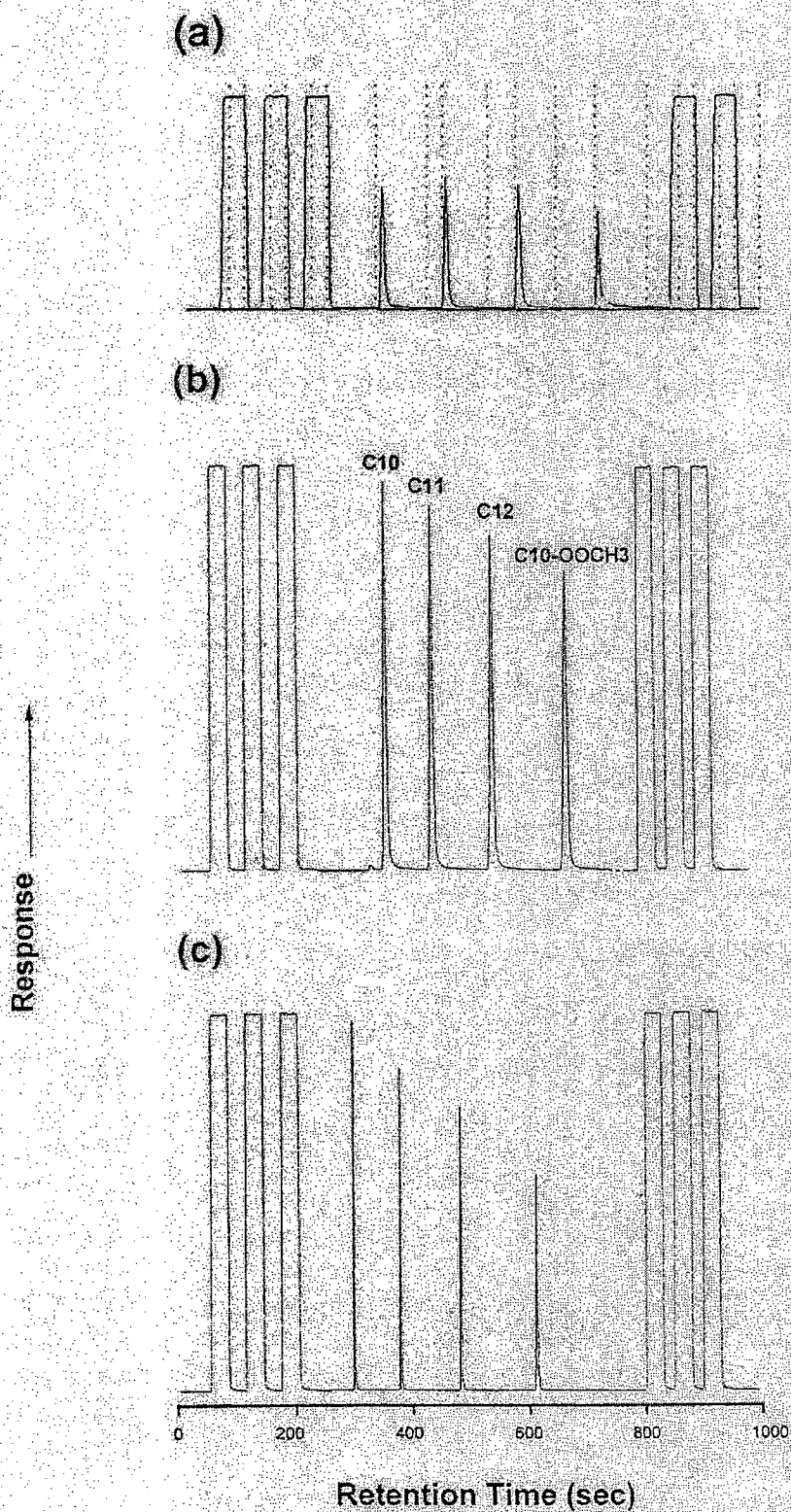


Fig. 4. irm-GC/MS selected ion chromatograms (m/z 44) showing VGMIx individual compound resolution obtained using standard commercially available glass tube furnace for (a) VG specification and (b) prior ARCO example in comparison to compound resolution obtained using (c) new ARCO centrifugal tube furnace.

Table 1. Diameters and volumes of commercially available ceramic and glass tube furnaces, in comparison to volumetric data obtained from capillary tube furnaces

| Tube ^a | Internal diameter (cm) | Volume of tube (cm ³) ^b | % Area reduction |
|-------------------|------------------------|--|------------------|
| Glass | 0.065 | 0.100 | 0 |
| Ceramic | 0.065 | 0.100 | 0 |
| Capillary | 0.053 | 0.066 | 34 |
| Capillary | 0.032 | 0.024 | 76 |
| Capillary | 0.025 | 0.015 | 85 |
| Capillary | 0.022 | 0.011 | 89 |
| Wire | 0.01 | 0.002 | N/A |

^aGlass and ceramic combustion tubes were purchased as 0.5 mm ID tubes; however scanning electron microscope measurements revealed actual tube dimensions to be of the order 0.65 mm ID [Fig. 3(b)]. ^bCalculated combustion tube volumes based on a reference 30 cm length. N/A means not applicable.

within the furnace, the polyimide-coating is removed from the tube along that portion of its length which will actually lie within the furnace, thus leaving an uncoated section of fused silica. Length(s) of catalytic wire (e.g. copper, nickel and/or platinum) are inserted into the capillary combustion tube and are sized to extend through that portion of tube situated within the furnace. Since the uncoated fused-silica capillary is potentially fragile, the capillary tube containing the metal oxide catalyst wires is placed within a glass or ceramic tube sheath for support. Direct capillary-to-capillary connections are then made using standard low or zero dead-volume capillary fittings, both to the column exiting the gas chromatograph, and, to the capillary column leading from the furnace to the isotope ratio mass-spectrometer via the water trap and open split.

The advantages of the capillary isotope furnace design are numerous. Firstly, the reduced volume of the capillary furnace reactor relative to existing commercial furnace reactors using larger bore glass or ceramic columns (Fig. 3), results in almost no loss in peak resolution to that obtained on compound separation upon exiting the gas chromatograph (Lambert *et al.*, 1984). Table 1 lists the internal diameters of standard glass and ceramic furnace tubes along with the internal area volumes related to an arbitrarily selected furnace tube of 30 cm length. The table also lists the internal area volumes associated with tube furnaces constructed from GC deactivated capillary tubing of 0.22, 0.25, 0.32 and 0.53 mm ID. The internal area volume taken up by standard metal oxide combustion wires is also noted for reference purposes. Using commercially obtained glass/ceramic furnace tube internal diameters as a reference (i.e. these represent the smallest commercially available furnace reactors), the percentage reduction in area volume associated with the use of progressively smaller furnace tube alternatives is shown. For example, the 0.53 mm ID capillary furnace tube represents a 34% volume reduction, while the 0.22 mm ID capillary furnace tube represents an 89% volume reduction over standard glass and ceramic furnace tubes. Clearly, significant improvements in area volume reduction can

be achieved through construction of a capillary tube furnace relative to standard glass and ceramic tube furnaces. Although the metal oxide combustion wires only occupy 0.002 cm³ of space each, our experience has indicated that only one wire should be used for the 0.22 or 0.25 mm ID constructed capillary tube furnaces, two wires for 0.32 mm ID constructed tube furnaces and three wires for 0.53 mm ID constructed tube furnaces. Use of more wires than suggested above appeared to result in a greater tendency for breakages in the uncoated glass capillary due to a "corkscrewing" effect the metal oxide wires adopted when routine maintenance required cooling of the furnace oven. Increases in column head pressure were also noted when additional wires were inserted, perhaps attributed to column obstructions resulting in back-pressure (Lambert *et al.*, 1984).

By far, the greatest improvement in the chromatography observed, resulted from the use of direct capillary-to-capillary zero dead-volume connections. The advantage of direct capillary-to-capillary connections, equates to fewer problems associated with gas leaks, caused by using fittings of substantially different sizes, as it permits the operator to use standard well proven leak tight capillary gas fittings. By providing a continuous capillary flow-path through the irm-GCMS unit, substantial amounts of dead-volume are eliminated, thereby increasing the peak resolution and enabling better recognition of peak start and peak end positions needed to accurately measure component isotope responses. The capillary columns themselves are cheap and standard to that obtainable from most laboratory equipment suppliers. The technique for constructing a capillary furnace reactor is simple and uses commonly available gas chromatographic materials.

Figure 4 shows three *m/z* 44 chromatograms of a reference mixture (VGmix, see experimental) provided by the instrument manufacturer that allows for comparisons in chromatography between standard tube furnace designs and the newly employed capillary tube furnace. Figure 4(a) shows a *m/z* 44 chromatogram of the VGmix analyzed under the instrument manufacturers specifications (chromato-

c20
c23

C₂₁ Monomethyl Alkane
Peak Resolution

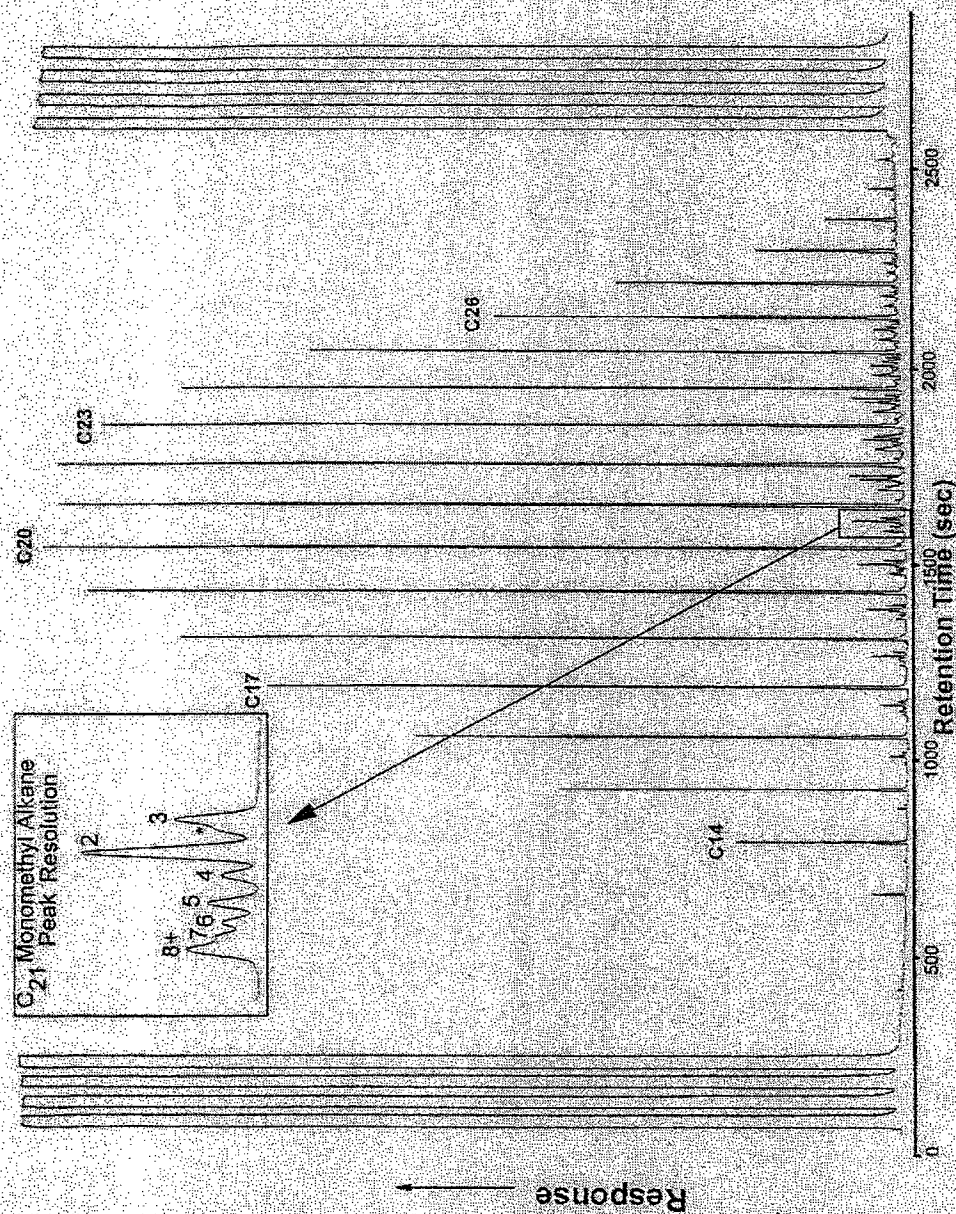


Fig. 5. irm-GC/MS selected ion chromatogram (m/z 44) showing enhanced compound resolution obtained with a "typical" petroleum sulfate fraction employing a capillary tube furnace (*some co-elution with C₂₁ ACH).

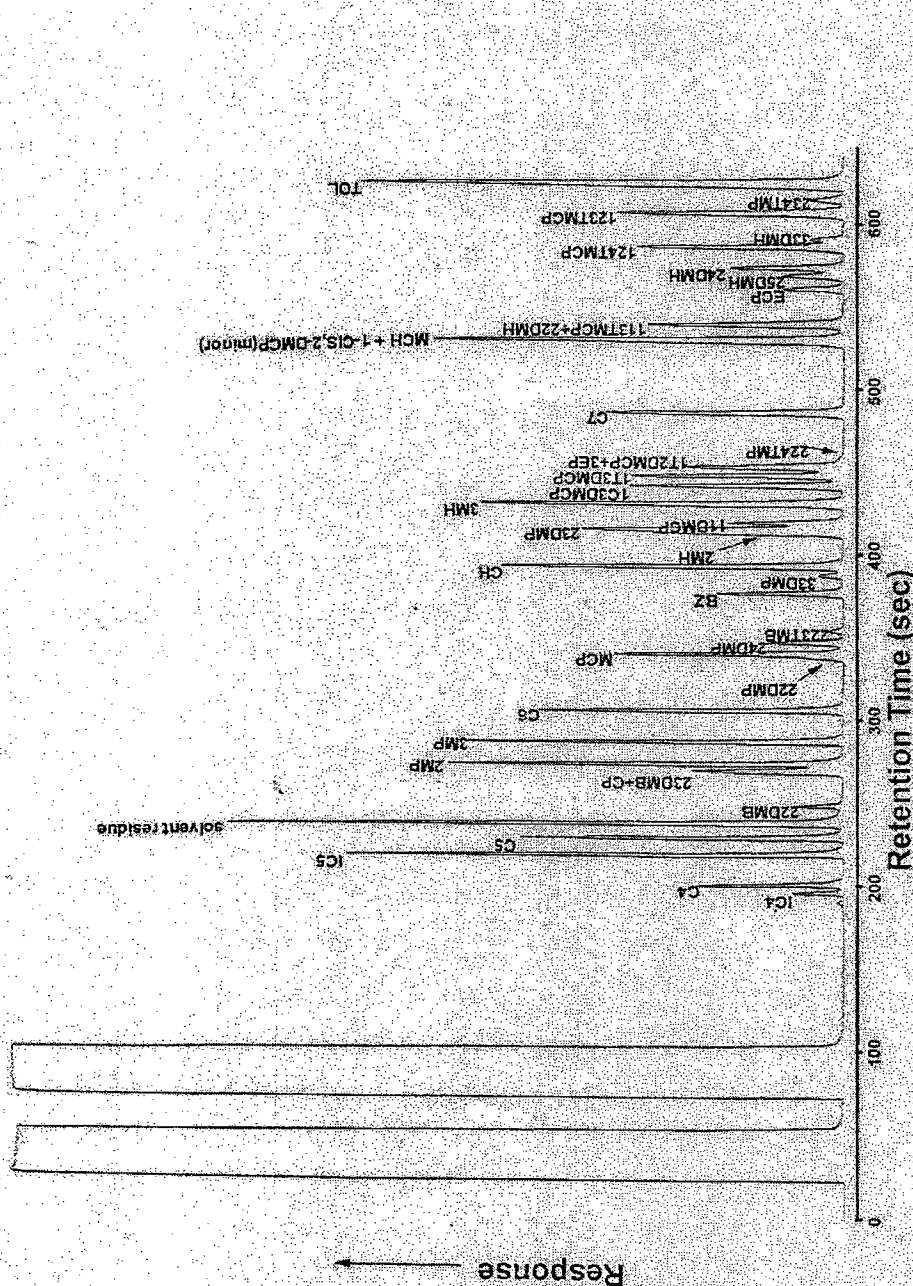


Fig. 6. Ion-GC/MS selected ion chromatogram (m/z = 44) showing compound resolution of C₇-C₉ gasoline components obtained from analysis of petroleum condensate example employing a capillary tube furnace. IC is isodane, DMB dimethylbutane, CP cyclopentane, MH methylhexane, DMCP dimethylcyclopentane, EP ethylpentane, MCP methylcyclopentane, TMB trimethylbutane, BZ benzene, CH cyclohexane, MH methylhexane, DMCP dimethylcyclopentane, EP ethylpentane, TMP trimethylpentane, MCH methylcyclohexane, TMCP trimethylcyclopentane, DMH dimethylhexane, ECP ethylcyclopentane, TMI trimethylpentane and TOL toluene.

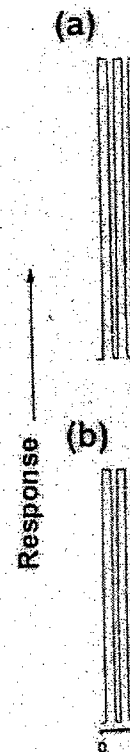


Fig. 7

gram taken 4(b) shows VGmix anal amic tube fu shows almo: with the V-instrument c chromatogra modified ca under identi obtained in capillary tub ements in c ution. The obtained up which great isotope mea the data. T modest imp ally result i individual c and interpr roleum frac cacy.

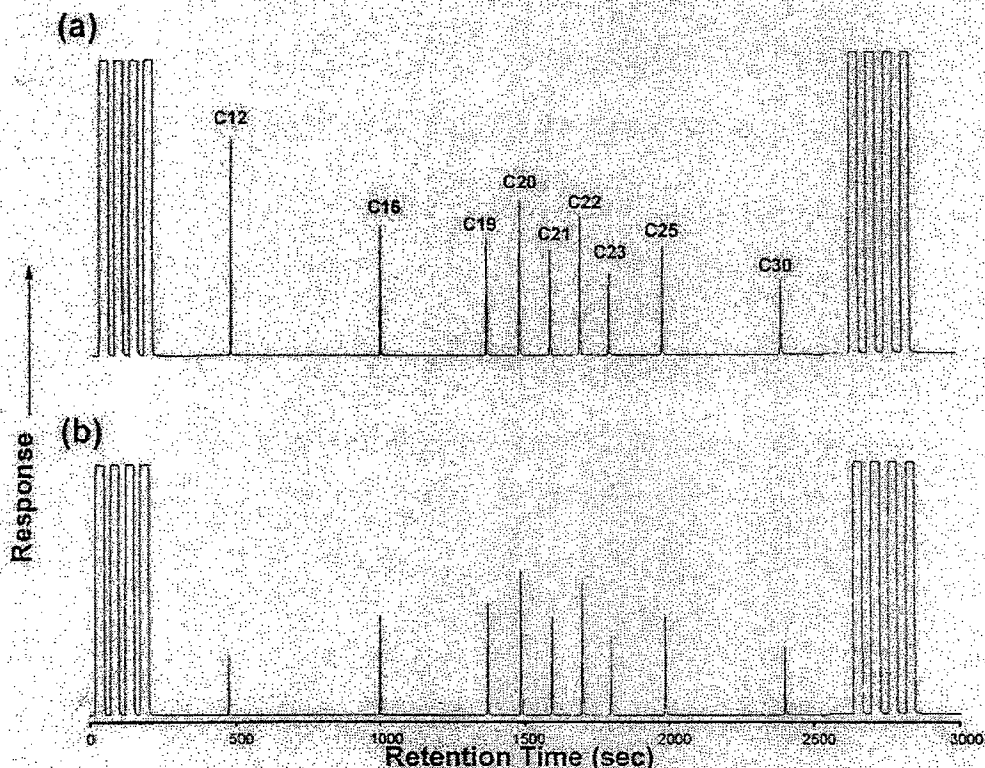


Fig. 7. irm-GC/MS selected ion chromatogram (m/z 44) showing comparison of chromatograms of (a) reference n -alkane mixture and (b) reference n -alkane mixture after urea adduction.

gram taken from VG reference manual). Figure 4(b) shows a m/z 44 chromatogram of the same VGmix analyzed "in-house" using a standard ceramic tube furnace. Comparison of Fig. 4(a) and (b) shows almost identical chromatography associated with the VG mix when analyzed under similar instrument conditions. Figure 4(c) shows a m/z 44 chromatogram of the VGmix making use of the modified capillary furnace design, and analyzed under identical run conditions to the chromatogram obtained in Fig. 4(b). Clearly, the introduction of a capillary tube furnace results in significant improvements in compound chromatography and resolution. The compound separation and resolution obtained upon exiting the GC oven is maintained, which greatly improves the determination of the isotope measurements and hence, the accuracy of the data. It is important to note, that seemingly modest improvements in peak resolution will actually result in large degrees of separations between individual components, thus facilitating the analysis and interpretation of complex mixtures such as petroleum fractions with greater confidence and efficacy.

Peak resolution

Peak resolution relating to compound separation and analysis is an essential requirement to achieve accurate compound specific isotope measurements (Meier-Augenstein, 1997). Effluent from a gas chromatograph capillary column connected to a glass or ceramic furnace tube will typically encounter a region of a substantially larger internal volume relative to the capillary column, resulting in peak broadening. Higher gas chromatograph head pressures creating faster column gas flows can result in narrower peak profiles (Lee *et al.*, 1984). However, this does not solve the fundamental problems related to peak resolution and compound separation, and any peak broadening effect or component co-elution caused by flow through a larger internal volume glass or ceramic tube furnace is permanent. The "sharper" and better resolved the peak on a chromatographic trace, the more accurate is the measurement of that particular carbon dioxide isotope signature (i.e. integrated area under the peak) since the exact points along the curve on the chromatographic trace at which a particular peak begins and ends (i.e. the points between which the curve is

Table 2. Comparison of measured isotope values obtained for a set of *n*-paraffin standards before and after treatment with urea clathration techniques

| Reference compound | Standard mixture | | Urea adducted | | % Adducted |
|---------------------------|---------------------------|--------------------|---------------------------|--------------------|------------|
| | $\delta^{13}\text{C}$ (‰) | σ ($n=3$) | $\delta^{13}\text{C}$ (‰) | σ ($n=3$) | |
| <i>n</i> -C ₁₂ | -32.3 | ±0.1 | -32.3 | ±0.1 | 30 |
| <i>n</i> -C ₁₆ | -29.2 | ±0.1 | -29.3 | ±0.1 | 88 |
| <i>n</i> -C ₁₉ | -28.7 | ±0.1 | -28.8 | ±0.1 | 96 |
| <i>n</i> -C ₂₀ | -43.6 | ±0.1 | -43.6 | ±0.1 | 100 |
| <i>n</i> -C ₂₁ | -28.6 | ±0.1 | -28.7 | ±0.1 | 100 |
| <i>n</i> -C ₂₂ | -28.7 | ±0.1 | -28.8 | ±0.1 | 100 |
| <i>n</i> -C ₂₃ | -26.7 | ±0.1 | -26.8 | ±0.1 | 100 |
| <i>n</i> -C ₂₄ | -28.0 | ±0.1 | -28.1 | ±0.1 | 100 |
| <i>n</i> -C ₂₆ | -29.2 | ±0.1 | -29.4 | ±0.1 | 100 |

to be integrated) can be more readily identified. Also, in more complex mixtures where two or more peaks elute close together, the "slower" and isotopically lighter component of one may be erroneously included in the data of the following peak (Hayes *et al.*, 1990). While these errors may appear to be small with respect to peak area, they nevertheless generate significant errors in the precise isotope calculations ultimately made from these measurements.

Examination of the literature reveals large variations in the resolution of *n*-alkane *m/z* 44 selected ion chromatograms of petroleum samples, as performed on different instruments in various laboratories (irm-GC/MS analysis is typically performed on saturate fractions of crude oil mixtures when paraffin isotope data are required for geochemical interpretations). Figure 5 shows a *m/z* 44 chromatogram of a urea adducted saturate fraction obtained from a crude oil mixture. It can be observed in Fig. 5 that excellent baseline resolution of the *n*-alkanes is obtained from the capillary combustion furnace. Resolution of components eluting between homologous *n*-alkanes, that include monomethyl and cycloalkane components can clearly be seen (see inset) and in some cases are also baseline resolved relative to other minor "interfering" components.

Other recent petroleum applications of irm-GC/MS relate to analysis of low molecular weight gasoline range (C₄-C₁₀) components (Bjorøy *et al.*, 1994). Figure 6 shows a *m/z* 44 selected ion chromatogram of C₄ to C₇ component resolution obtained from analysis of a petroleum condensate. As with Fig. 5, excellent baseline separation of many of the gasoline range components is obtained through use of a capillary furnace and compound resolution is directly comparable to that obtained from standard high resolution GC analysis.

Size-exclusion techniques

In order to assess the viability of chromatography techniques such as urea adduction and molecular sieve separations for irm-GC/MS applications, the question of introduced isotopic fractionation must

first be addressed. While, in general, no isotopic fractionation of *n*-alkanes isolated using urea adduction has been reported in the literature, there exists little isotope data in relation to the lower molecular weight series (<C₂₀) of *n*-alkanes that are not quantitatively adducted. To investigate this issue, a standard mixture of commercially available C₁₂-C₂₆ *n*-alkanes was prepared and urea adduction sample preparation applied.

Figure 7 shows *m/z* 44 chromatograms for the standard paraffin mixture [Fig. 7(a)], together with the same mixture after urea adduction [Fig. 7(b)]. Investigations in this work found that the technique of urea adduction fails to completely adduct *n*-alkane compounds with less than 20 carbons. This introduces compound fractionation and therefore also the possibility of isotope fractionation. Table 2 lists the background subtracted measured isotope values for the standard paraffin mixture and also the measured isotope values for the standard paraffin components after urea adduction. From these data, it is clear that no isotope fractionation of the paraffin components in the standard mixture, including those that were only partially adducted (e.g. C₁₂, C₁₆ and C₁₉), has occurred. Reproducibility of the measured isotopes was less than 0.1‰ in all cases. Evidence is presented, therefore confirming that isotopic fractionation was not observed to occur with the use of urea adduction sample preparation techniques in this study.

To examine the effect that other co-eluting components or the background matrix may have on the measurement of individual isotope values for paraffins in a typical crude oil, a comparison of data acquired from irm-GC/MS analysis of a whole crude oil with that of both a urea adducted and molecular sieved sample was undertaken. Figure 8(a) shows a *m/z* 44 chromatogram of a light crude oil analyzed directly or "neat" with no sample preparation. This chromatogram shows a dominant series of *n*-alkanes with a carbon distribution ranging from C₁₁ to C₃₀. The sample, although unaffected by biodegradation or PVT migration/evaporation effects, does show a slightly elevated baseline reflecting an unresolved complex mixture



Fig. 8. irm-GC light crude oil

or background observed in most *m/z* 44 chromatogram rate fraction after

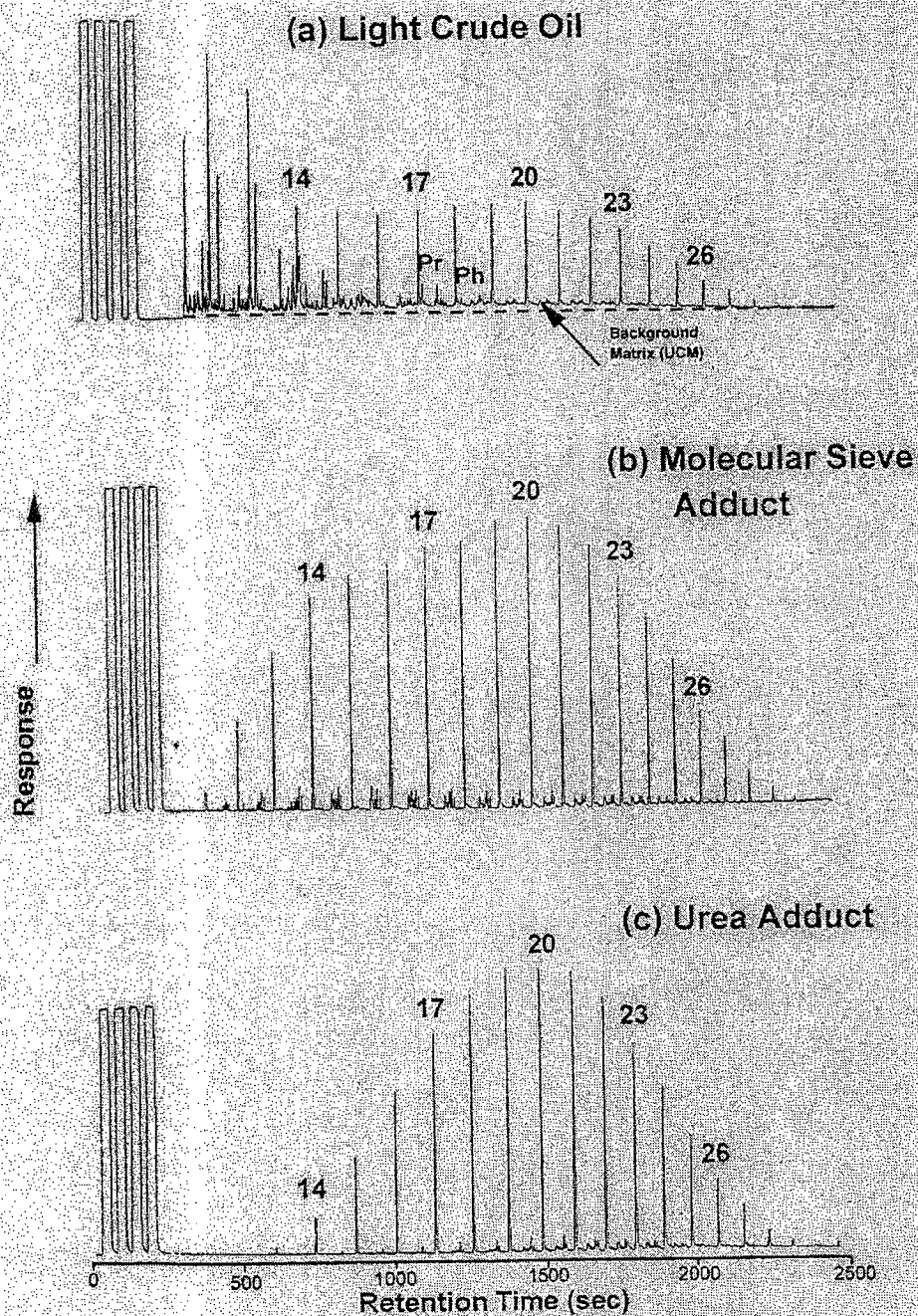


Fig. 8. irm-GC/MS selected ion chromatogram (m/z 44) showing comparison of chromatograms of (a) light crude oil ("neat"), (b) molecular sieve adducted fraction of saturated components and (c) urea adducted fraction of saturated components.

or "background matrix", that is commonly observed in most crude oils. Figure 8(b) shows a m/z 44 chromatogram of the same light crude oil saturate fraction after treatment with molecular sieves

Similarly to urea adduction, molecular sieving fails to completely adduct paraffin components with less than 20 carbons (Ellis, 1989; West *et al.*, 1990). Figure 8(c) shows a m/z 44 chromatogram of the

Table 3. Comparison of urea and molecular sieving adduction efficiencies for *n*-alkanes in a crude oil

| Compound | Urea (%) | Molecular sieving (%) |
|-----------------------------|----------|-----------------------|
| <i>n</i> -C ₁₃ | 3 | 66 |
| <i>n</i> -C ₁₄ | 13 | 81 |
| <i>n</i> -C ₁₅ | 37 | 92 |
| <i>n</i> -C ₁₆ | 61 | 96 |
| <i>n</i> -C ₁₇ | 81 | 98 |
| <i>n</i> -C ₁₈ | 90 | 97 |
| <i>n</i> -C ₁₉ | 94 | 96 |
| <i>n</i> -C ₂₀ | 100 | 100 |
| <i>n</i> -C ₂₁ + | 100 | 100 |

urea adducted saturate fraction of the same light crude oil shown in Fig. 8(a). Table 3 lists the comparative efficiencies for *n*-alkane adduction between the two sample preparation techniques, and shows molecular sieving techniques can be observed to adduct a greater proportion of the paraffin components below *n*-C₂₀ relative to urea sample treatment in this case. Both adduction sample

preparation techniques also isolate monomethyl, one-ring cyclic and in some cases dimethyl alkyl paraffins (Rubinstein and Strausz, 1979; Hoering and Freeman, 1984; Ellis, 1989). One notable chromatographic feature observed in Fig. 8(b) and (c) is the absence of an elevated baseline, suggesting the adduction sample preparation techniques also worked effectively in removing co-eluting components that typically comprise a background matrix/unresolved complex mixture (Killops and Al-Juboori, 1990; Ellis *et al.*, 1994).

Table 4 lists the measured isotope values for the *n*-alkane components in the "neat" oil analysis along with those data obtained from the same *n*-alkane components after saturate fraction isolation via column chromatography and subsequent sample treatment with molecular sieving and urea adduction techniques. Comparison of those data for *n*-alkane components isolated from the molecular sieve and urea adducted sample fractions reveals a

Table 4. Comparison of measured isotope values obtained for linear paraffins in a light crude oil in relation to those data obtained after treatment with urea elutriation and molecular sieving techniques

| Compound | Light crude oil | | Light crude oil saturate fraction | | Light crude oil molecular sieve adduct | | Light crude oil urea adduct | |
|---|---------------------------|--------------------|-----------------------------------|--------------------|--|--------------------|-----------------------------|--------------------|
| | $\delta^{13}\text{C}$ (‰) | σ ($n=3$) | $\delta^{13}\text{C}$ (‰) | σ ($n=3$) | $\delta^{13}\text{C}$ (‰) | σ ($n=3$) | $\delta^{13}\text{C}$ (‰) | σ ($n=3$) |
| <i>n</i> -C ₁₀ | -24.1 | ±0.0 | | | | | | |
| <i>n</i> -C ₁₁ | -27.0 | ±0.3 | -25.2 | ±0.1 | | | | |
| <i>n</i> -C ₁₂ | -25.9 | ±0.3 | -26.8 | ±1.3 | -25.6 | ±0.3 | | |
| <i>n</i> -C ₁₃ | -25.1 | ±0.3 | -25.0 | ±0.3 | -25.8 | ±0.0 | | |
| <i>n</i> -C ₁₄ | -23.5 | ±0.8 | -24.8 | ±0.2 | -24.0 | ±0.0 | -25.2 | ±0.2 |
| <i>n</i> -C ₁₅ | -33.0 | ±1.9 | -26.1 | ±0.1 | -25.8 | ±0.0 | -26.2 | ±0.1 |
| <i>n</i> -C ₁₆ | -24.4 | ±0.2 | -25.6 | ±0.1 | -23.6 | ±0.1 | -25.8 | ±0.2 |
| <i>n</i> -C ₁₇ | -26.6 | ±0.3 | -25.0 | ±0.2 | -25.5 | ±0.1 | -26.0 | ±0.2 |
| <i>n</i> -C ₁₈ | -25.8 | ±0.1 | -23.4 | ±0.1 | -25.6 | ±0.0 | -26.0 | ±0.4 |
| <i>n</i> -C ₁₉ | -25.4 | ±0.1 | -27.9 | ±0.3 | -25.8 | ±0.0 | -25.9 | ±0.2 |
| <i>n</i> -C ₂₀ | -26.0 | ±0.1 | -26.0 | ±0.1 | -25.7 | ±0.1 | -25.8 | ±0.2 |
| <i>n</i> -C ₂₁ | -25.4 | ±0.2 | -25.9 | ±0.1 | -25.7 | ±0.1 | -25.8 | ±0.2 |
| <i>n</i> -C ₂₂ | -25.7 | ±0.2 | -25.7 | ±0.1 | -25.7 | ±0.1 | -25.8 | ±0.2 |
| <i>n</i> -C ₂₃ | -25.6 | ±0.1 | -25.8 | ±0.1 | -25.7 | ±0.1 | -25.7 | ±0.3 |
| <i>n</i> -C ₂₄ | -25.2 | ±0.2 | -25.8 | ±0.1 | -25.8 | ±0.1 | -25.8 | ±0.3 |
| <i>n</i> -C ₂₅ | -26.5 | ±0.2 | -25.8 | ±0.2 | -25.7 | ±0.3 | -25.5 | ±0.3 |
| <i>n</i> -C ₂₆ | -25.0 | ±0.2 | -27.0 | ±0.3 | -25.9 | ±0.2 | -26.2 | ±0.3 |
| <i>n</i> -C ₂₇ | -25.1 | ±0.4 | -25.3 | ±0.2 | -25.5 | ±0.2 | -25.4 | ±0.3 |
| <i>n</i> -C ₂₈ | -25.5 | ±0.0 | -25.3 | ±0.4 | -25.3 | ±0.2 | -26.8 | ±1.0 |
| <i>n</i> -C ₂₉ | | | -25.8 | ±0.3 | -25.4 | ±0.1 | -25.4 | ±0.3 |
| <i>n</i> -C ₃₀ | | | -24.9 | ±0.0 | -25.5 | ±0.6 | -25.1 | ±0.3 |
| | | | | | -25.8 | ±0.7 | | |
| Internal Standard | | | | | | | | |
| d34-C16M | * | | -29.59 (±0.43) | | -28.67 (±0.12) | | -28.35 (±0.16) | |
| d34-C16R | -28.68 | | -28.68 | | -28.68 | | -28.68 | |
| | UA-oil (‰) | UA-sat (‰) | UA-MS (‰) | | | | | |
| Measured isotope value variability compared to urea reference | | | | | | | | |
| No internal standard correction | | | | | | | | |
| Mean | 1.1 | 0.5 | 0.3 | | | | | |
| Std. Dev. | ±2.12 | ±0.55 | ±0.36 | | | | | |
| Internal standard correction | | | | | | | | |
| Mean | * | 1.3 | -0.2 | | | | | |
| S.D. | * | ±0.79 | ±0.46 | | | | | |

*Internal standard corrections not applied due to observed co-elutions with deuterated standard in whole oil analysis. "d34-C16M": Measured carbon isotope value for co-injected *n*-C₁₆ internal standard; "d34-C16R": Reference carbon isotope value for *n*-C₁₆ internal standard; "oil": whole oil; "sat": saturate fraction; "MS": molecular sieve adduct; "UA": urea adduct.

n-C₁₆

Fig. 9. Intermatograms: frac

very close match components except for than 0.7‰ between value for *n* anomalous, since compound in the rate fraction and molecular sieving purity of comparison within the instrument agreement of the

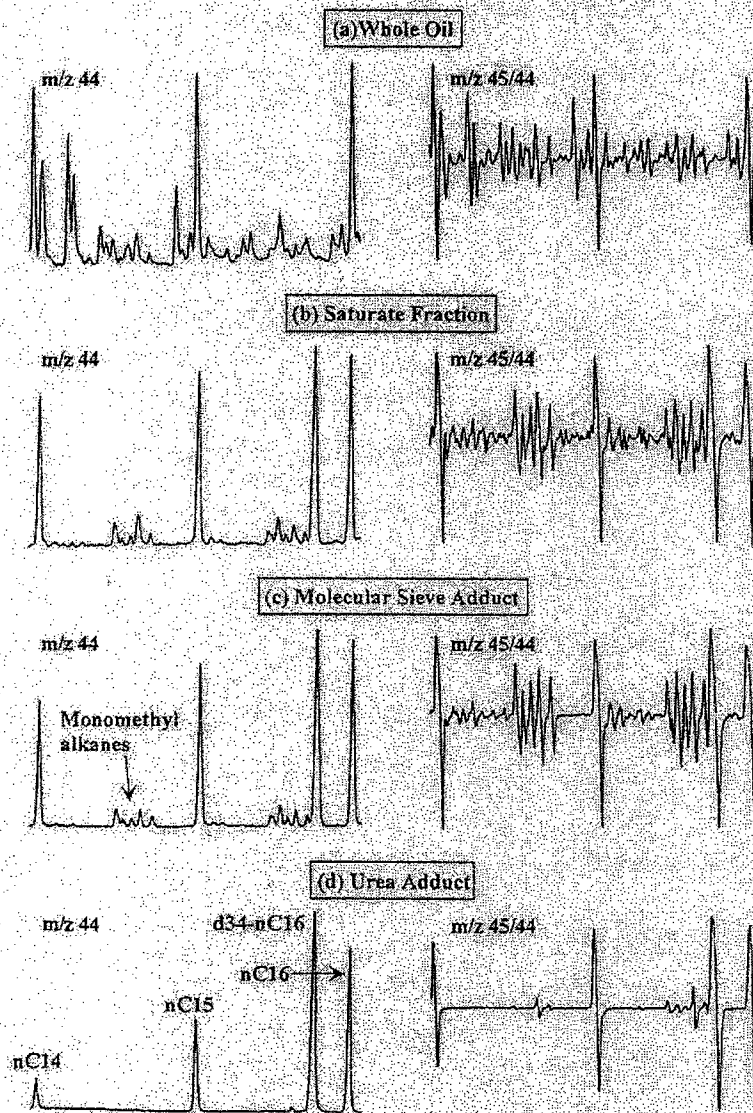


Fig. 9. irm-GC/MS selected ion chromatograms (m/z 44 and m/z 45/44) showing comparison of chromatograms of (a) light crude oil ("neat"), (b) isolated saturate fraction, (c) molecular sieve adducted fraction of saturated components and (d) urea adducted fraction of saturated components.

very close match with all individual sample components except one ($n\text{-C}_{15}$), differing by no more than 0.7‰ between the fractions. The measured isotope value for $n\text{-C}_{15}$ in the urea adduct is clearly anomalous, since measured isotope values for this compound in the corresponding whole oil and saturate fraction analysis correlate well with that of the molecular sieving measured isotope value. The majority of components (9 of 16) however, agreed to within 0.2‰ on average, a value considered well within the instrument precision error. The close agreement of the measured isotope values between

both adduction techniques indicates that molecular sieving, as previously shown with urea adduction (Table 2), shows no measurable isotope fractionation effect.

Isotopic analysis of paraffins in urea and molecular sieve adduct fractions should, therefore, provide analyses less affected by co-elutions problems. Measured isotope values for sample components in these fractions should be of higher overall precision and accuracy as compared to a whole oil analysis of the same sample. Employing measured isotope values for individual n -alkanes in the urea adduct

urea adduct/molecular sieve adduct
 <saturate<whole oil fraction

Techniques used to increase the accuracy of an isotope measurement may include the addition of an internal standard of known isotopic composition (Merritt *et al.*, 1994). Commonly for petroleum applications, a perdeuterated *n*-alkane is preferred, which has the advantage of never co-eluting with an *n*-alkane component of interest and also exhi-

Although crude oil compositions vary considerably from sample to sample, the examples shown here are intended to reflect general features typically observed with most oils, such as a predominance of *n*-alkanes between C_{10} and C_{30} and a low-level background matrix or UCM. These characteristics, are in fact, those usually associated with "good quality" oils, whereas quite often crude oil mixtures may be substantially less "clean" in comparison, due to effects of biodegradation, low maturity and variable source quality. Considering the intensive effort and expense in generating firm GC/MS data, the use of relatively inexpensive chromatography techniques such as urea adduction and molecular sieving in isolating components of interest for accurate isotope analysis would appear to be justified.

Introduction of a capillary tube furnace in place of standard commercially obtained glass ceramic tube furnaces has resulted in significant improve-

Acknowledgements—mission to publish Liang for his SEM reviews of the n Summons and J. l ance. The authors K. Hall and C. Bo providing many h contributed to imp

- Bjorøy, M., Hall, in the isotopic α - $C_{2}-C_{20}$ fraction
Geochemistry 21,
Brenna, J. T. (1987) mass-spectrometry and biomedical
Research 27, 340
Ellis, L. (1989) No isolated from Honours disserta
Ellis, L., Alexandre of petroleum by nile molecular si
nanthrenes. *Org*
Ellis, L. and Finca
ments in im-GC
design and sam
17th Intl. mee
Forschungszentr
Goodman, K. J. in restoration of a chromatography, trome try. *Anal*
Hayes, J. M., Free C. H. (1990) C novel tool for re processes. *Org*

ments in chromatographic resolution of complex petroleum mixtures analyzed using irm-GC/MS techniques. Application of urea adduction and molecular sieve sample preparation techniques to complex petroleum mixtures has been shown to provide "simpler" petroleum fractions containing fewer components concomitant with less co-elution problems. No isotope fractionation was observed using the urea adduction and molecular sieve sample preparation techniques. Use of a capillary furnace tube together with application of sample preparation techniques has resulted in significant improvements in both the precision and accuracy of measured isotope values in irm-GC/MS analysis of complex petroleum mixtures.

Acknowledgements—The authors thank ARCO for permission to publish. We would also like to thank Long Liang for his SEM analysis and Albert Holba for internal reviews of the manuscript. Many thanks to Dr R. Summons and J. Hope (AGSO) for analyses and assistance. The authors would also like to acknowledge Drs K. Hall and C. Boreham for reviewing the manuscript and providing many helpful suggestions, which have greatly contributed to improving the quality of this paper.

REFERENCES

- Bjorøy, M., Hall, P. B. and Moe, R. P. (1994) Variation in the isotopic composition of single components in the C_{15} – C_{20} fraction of oils and condensates. *Organic Geochemistry* 21, 761–776.
- Brenna, J. T. (1994) High-precision gas isotope ratio mass spectrometry: recent advances in instrumentation and biomedical applications. *Accounts in Chemical Research* 27, 340–346.
- Ellis, L. (1989) Novel homologous series of hydrocarbons isolated from petroleum using molecular sieves. Honours dissertation, Curtin University Tech.
- Ellis, L., Alexander, R. and Kagi, R. I. (1994) Separation of petroleum hydrocarbons using dealuminated mordenite molecular sieve II. Alkyl-naphthalenes and alkylphenanthrenes. *Organic Geochemistry* 21, 849–855.
- Ellis, L. and Fincannon, A. L. (1997) Analytical improvements in irm-GC/MS analyses: advances in tube furnace design and sample preparation. In *Abstracts from the 17th Intl. Meet. Org. Geochem.*, Maastricht 1997. Forschungszentrum Jülich, pp. B86–87.
- Goodman, K. J. and Brenna, J. T. (1994) Curve fitting for restoration of accuracy for overlapping peaks in gas chromatography/combustion isotope ratio mass spectrometry. *Analytical Chemistry* 66, 1294–1301.
- Hayes, J. M., Freeman, K. H., Popp, B. N. and Hoham, C. H. (1990) Compound-specific isotopic analyses: a novel tool for reconstruction of ancient biogeochemical processes. *Organic Geochemistry* 16, 1115–1128.
- Hoering, T. C. and Freeman, D. H. (1984) Shape selective sorption of monomethyl alkanes by silicalite, a zeolitic form of silica. *Journal of Chromatography* 316, 333–341.
- Killops, S. D. and Al-Juboori, M. A. H. A. (1990) Characterisation of the unresolved complex mixture (UCM) in the gas chromatograms of biodegraded petroleum. *Organic Geochemistry* 15, 147–160.
- Lambert, B. J., Laub, R. J., Roberts, W. L. and Smith, C. A. (1984) Reduction of the column diameter in capillary gas chromatography: Some theoretical aspects and practical consequences. In *Ultrahigh Resolution Chromatography*, ed. S. Ahuja. ACS Symposium Series 250, pp. 49–58.
- Lee, M. L., Yang, F. J. and Bartle, K. D. (1984) *Open Tubular Column Gas Chromatography: Theory and Practice*. Wiley, New York.
- Matthews, D. E. and Hayes, J. M. (1978) Isotope-ratio-monitoring gas chromatography-mass spectrometry. *Analytical Chemistry* 50, 1465–1473.
- Majer-Augenstein, W. (1987) The chromatographic side of isotope ratio mass spectrometry: pitfalls and answers. *LC-GC* 15, 244–253.
- Merritt, D. A. and Hayes, J. M. (1994) Factors controlling precision and accuracy in isotope-ratio-monitoring mass spectrometry. *Analytical Chemistry* 66, 2336–2347.
- Merritt, D. A., Brand, W. A. and Hayes, J. M. (1994) Isotope-ratio-monitoring gas chromatography-mass spectrometry: methods for isotopic calibration. *Organic Geochemistry* 21, 573–583.
- Merritt, D. A., Freeman, K. H., Ricci, M. P., Studley, S. A. and Hayes, J. M. (1995) Performance and optimization of a combustion interface for isotope ratio monitoring gas chromatography-mass spectrometry. *Analytical Chemistry* 67, 2461–2473.
- Murphy, M. T. J. (1969) Analytical Methods. In *Organic Geochemistry*, ed. G. Eglington and M. T. J. Murphy. Springer, Berlin, pp. 74–88.
- Nolte, D. G. (1991) Separation of a mixture of normal paraffins, branched chain paraffins and cyclic paraffins. U.S. Patent #4,982,052.
- Ricci, M. P., Merritt, D. A., Freeman, K. H. and Hayes, J. M. (1994) Acquisition and processing of data for isotope-ratio-monitoring mass spectrometry. *Organic Geochemistry* 21, 561–571.
- Rubinstein, I. and Strausz, O. P. (1979) Geochemistry of the thiourea adduct fraction from an Alberta petroleum. *Geochimica et Cosmochimica Acta* 43, 1387–1392.
- Sofer, Z., Bjorøy, M. and Hustad, E. (1991) Isotopic composition of individual *n*-alkanes in oils. In *Organic Geochemistry: Advances and Applications in Energy and the Natural Environment*, ed. D. A. C. Manning. Manchester University Press, pp. 207–211.
- West, N., Alexander, R. and Kagi, R. I. (1990) The use of silicalite for rapid isolation of branched and cyclic alkane fractions of petroleum. *Organic Geochemistry* 15, 499–501.
- Wong, W. W., Hachey, D. L., Zhang, S. and Clarke, L. L. (1995) Accuracy and precision of gas chromatography/combustion isotope ratio mass spectrometry for stable carbon isotope ratio measurements. *Rapid Communications in Mass Spectrometry* 9, 1007–1011.

Review

Isotopic Fractionation of Organic Compounds in Chromatography

Crist N. Filer

NEN Life Science Products
549 Albany Street
Boston, Massachusetts 02118

I. Introduction

During the past fifty years the development of synthetic methods to prepare and analytical methods to detect isotopically labeled organic compounds has made them exceedingly valuable tools for research. Stable isotopes such as ^2H , ^{13}C and ^{15}N have been used to elucidate biosynthetic schemes and reaction mechanisms, and radioisotopes such as ^3H , ^{14}C , ^{32}P , ^{35}S , and ^{125}I have been exploited to clarify metabolic pathways, facilitate biological assays and deduce the structure of complex molecules like DNA.

After the synthetic preparation of an isotopically substituted organic compound it is a typical practice to corroborate isotopic product identity by performing cochromatography (demonstration of the same R_f or retention time) with an authentic nonlabeled standard in multiple chromatographic systems. This exercise is especially crucial for radioisotopes like tritium where the tiny mass of product involved often precludes alternative identity confirmation such as IR, NMR and the like. It is our experience that for the vast majority of cases such isotopically labeled compounds completely cochromatograph with authentic nonlabeled substances. However, for over forty years, some interesting and well documented examples have emerged for all types of chromatography where isotopically labeled compounds and authentic unlabeled standards simply do not cochromatograph. In fact in some cases dramatic baseline peak resolution can be achieved. This

chromatographic phenomenon has been referred to as isotopic fractionation and was last reviewed by Klein (1) over thirty years ago. In this review the phrase "isotopic fractionation" refers only to chromatographic isotopic fractionation. Since Klein's review a partial discussion of the topic has appeared in several other limited publications (2-5).

The purpose of this discussion will be to review the documented literature instances of chromatographic fractionation for isotopically labeled organic compounds, especially the many intriguing examples that have been published since Klein's first review. It excludes a number of unpublished instances of the phenomenon for compounds confidential to us or those we have collaborated with. This summary was presented in part at a recent Philadelphia International Isotope Society (IIS) meeting (6) and the cited papers usually fall within two categories. Some of them are totally devoted to an observation of isotopic fractionation often convincingly documenting it, exploring its scope, proposing an explanation or mechanism for its occurrence and placing it in context with other examples. However, many cited authors in diverse disciplines often disclose a single well characterized observation of isotopic fractionation as a curious side issue to the main body of their work. It is clear that some of these investigators were unaware of the phenomenon prior to their own observation and sometimes surprised by it. This review most likely captures all of the pertinent references on isotopic fractionation from 1955 to the end of 1997, but has excluded other articles whose observation or claim of the phenomenon was somewhat ambiguous. Articles were located not only by a painstaking and meticulous review of key chromatographic journals (tables of contents and indices) since 1955, but also by exhaustive cross reference checking of retrieved articles and citation searching of past papers to uncover later ones referencing them. The chromatographic techniques covered include gravity column, ion exchange, electrophoresis, paper, thin layer, gas and high pressure liquid chromatography. Although there are several reports of isotopic fractionation for the technique of countercurrent distribution (7-9), this topic will not be addressed here. Finally, the proposed factors causing chromatographic peak separation and its implications will be reviewed. Armed with this information, investigators in many life science areas using labeled

compounds will be better prepared to anticipate and interpret such results if they occur in their own laboratories.

II. Column Chromatography

Perhaps the first mention of isotopic fractionation for an organic compound in the chemical literature was by Van Dyken in the abstracts of a 1955 American Chemical Society meeting in Minneapolis (10) and later in a larger paper where the same data was incorporated and discussed (11). It was observed that on a silica gel column eluted with mixtures of butanol:chloroform, tritium labeled acids like formic acid manifested separation from their unlabeled standards. In most cases the tritiated acid eluted later than the unlabeled analogue as shown by specific activity (the measurement of radioactivity per unit of mass, often expressed as mCi/mmol) determinations of successive peak cuts.

Isotopic fractionation has been observed on column chromatography not only for such small molecules but also for much larger ones like the steroids [acetate-2-³H] cholesterol acetate (12-14), [1,2-³H] aldosterone (15-19), [³H] 7-dehydrocholesterol (20), [1,2,6,7-³H] testosterone (21), a tritiated ecdysone related compound (22) and lipids like [³H] methyl linoleate (23). In many of these examples the coelution of analogues labeled separately with carbon-14 and tritium was examined by measuring the changing ratio of ¹⁴C/³H on successive peak cuts during chromatography. A great deal of effort was expended in ruling out impurities and other chromatographic artifacts as alternative explanations for the observations since many of these early reports were still met with relative disbelief (1). From these early studies several conclusions were already drawn about the phenomenon in the context of column chromatography. Tritium more often than carbon-14 appeared to cause isotopic fractionation and its presence almost always retarded compound elution on silica gel compared to the unlabeled substance. Also, the location of tritium labeling appeared to be a strong factor in the mechanism and extent of isotopic fractionation. Finally, it was appreciated that the degree of isotopic fractionation could be effected by column conditions.

Several examples of isotopic fractionation with stable isotope labeled organic compounds by column chromatography have been reported. A deuterium labeled

prostaglandin showed isotopic fractionation during column chromatography but its oxygen-18 analogue did not and use of the latter circumvented this "undesirable" property (24). Carotenoids labeled with deuterium could be separated from their unlabeled counterparts on activated magnesium oxide (25) and in this instance the deuterated compound migrated more slowly. Finally, several deuterated p-xylenes were separated by column chromatography from unlabeled analogues by cleverly taking advantage of a differential host/guest mechanism with tetrakis(4-methylpyridine)nickel (II) thiocyanate that had previously been employed to separate nonlabeled aromatic isomers (26). This appears to be the first report utilizing such an inclusion process to promote isotopic fractionation, but surprisingly the authors stopped short of suggesting what would seem to be a possible steric explanation (see Section IX).

III. Ion Exchange Chromatography

Although obviously related to column chromatography, ion exchange chromatography is unique enough to merit separate discussion. A very systematic study of isotopic fractionation as influenced by isotope location was the work of Piez and Eagle (27, 28) using ion exchange chromatography on several amino acids labeled with carbon-14. Elution of a Dowex 50 column with a pH gradient clearly demonstrated that several carbon-14 labeled amino acids migrated more slowly on the column than their unlabeled counterparts. It was evident from even this early work that the increased molecular weight of the radiolabeled compound alone could not satisfactorily explain the observed degree of isotopic fractionation, and it was also apparent that the position of the radiolabel in the amino acid had a marked effect on its mobility. In general, the greatest amount of isotopic fractionation was noted when carbon-14 was adjacent to the amine. Since this initial observation, other related instances of carbon-14 amino acid isotopic fractionation by ion exchange chromatography have been reported (29-33), discussing both the mechanism of the phenomenon and its implications. A separation was also described for [^{14}C] formic acid (34) via ion exchange chromatography on a strongly basic Dowex-2 resin. In this latter example the carbon-14 labeled compound was significantly enriched in the earlier eluent.

Several compounds labeled with tritium have been reported to show isotopic fractionation in this medium. [^3H] 2-Aminopurine eluted earlier than authentic unlabeled standard on a Dowex 1-X8 column (35). Special care was taken to exclude impurities as the cause of isotopic fractionation in this instance by paper chromatography analysis of fractions at the front and back of the radioactive peak. It was found that both the early and late eluting peak fractions were homogeneous and cochromatographed with authentic 2-aminopurine. Noting that the degree of isotopic fractionation was influenced by eluent pH, this report was also one of the first to implicate the influential role of tritium in modifying the polarity of an adjacent nitrogen atom. Several reports on the isotopic fractionation of tritium labeled amino acids by ion exchange chromatography have appeared (36, 37). It was observed that unlike carbon-14, which almost always resulted in a less mobile amino acid, the retention time of an amino acid labeled with tritium was very dependent on the radiolabel location. On a Dowex 1-X4 column, several carbohydrates including [$6\text{-}^3\text{H}$] maltose (38) and [$6\text{-}^3\text{H}$] D-glucose also exhibited isotopic fractionation (39). While there was only a slightly increased compound mobility in the former example, in the latter it was significant enough for the authors to speculate that with early fraction collection and recycling, very isotopically pure [$6\text{-}^3\text{H}$] D-glucose could be obtained. Also, the separation of tritium labeled folate derivatives from carbon-14 and unlabeled folate analogues (40) as well as the earlier elution of [^3H] ethylenediamine-ouabain (41) by ion exchange chromatography have been described.

A few reports have related the ion exchange isotopic fractionation of stable isotope containing organic compounds. [^{15}N] Urea was enriched in isotopic content by means of its isotopic fractionation on cation exchange chromatography (42) and several deuterated glucose isomers were separated by anion exchange chromatography (43). In this latter example using two CarboPac PA1 columns in series, baseline separation was realized for [$1\text{-}^2\text{H}$] D-glucose (earlier eluting) and unlabeled D-glucose. Also by this method almost baseline separation was achieved for [$1\text{-}^3\text{H}$] D-glucose (earlier eluting) from [$2\text{-}^3\text{H}$] D-glucose. The authors concluded that these results rule out mass or hydrophobicity as being operative in the separation. Rather, they proposed that the inductive effect of the [$1\text{-}^2\text{H}$] D-glucose

C-²H bond reduces the adjacent C-O bond strength thereby increasing the adjacent O-H bond strength making it less acidic than in the case of the [2-²H] D-glucose.

IV. Electrophoresis

Only a few examples of isotopic fractionation by means of electrophoresis have been described but several have been fairly striking. Giovanelli and coworkers reported the significant isotopic fractionation of [³H] GSH sulfonic acid (44). With regard to stable isotopes, [²H] dansylated methylamine separated with almost baseline resolution as a faster peak from unlabeled compound via micellar electrokinetic capillary chromatography (45, 46). In this study the addition of methanol to the mobile phase, use of long capillaries, high voltages and a stepped application of the voltage were key contributing factors to the successful separation observed.

Recently, Chiari described the complete separation of several deuterated compounds (such as [²H] aniline, [²H] benzoic acid and [²H] pyridine) from unlabeled compounds by capillary zone electrophoresis (47). The mechanism suggested for this fractionation was that the electron donating inductive effect of ring deuterium (compared to hydrogen) altered the ionization constant of nearby functionality. Using this same technology, Terabe and coworkers were also able to completely resolve unlabeled benzoic acid (earliest eluting) from single (middle eluting) and double (last eluting) labeled [¹⁸O] benzoic acid isotopomers (48). The authors optimized applied voltages, capillary tube lengths, buffer pH and electroosmotic velocities to achieve this stunning separation and proposed that this method was more efficient than HPLC to obtain the resolution of such closely related ionizable compounds.

V. Paper Chromatography

To date, instances of isotopic fractionation reported for paper chromatography have been few. Several deuterated amino acids and carbohydrates showed partial separation from authentic standards in this medium (49, 50). In both cases the deuterated compounds migrated more slowly than the unlabeled substances. Other examples include the separation of tritiated steroids

from cold or carbon-14 analogues during paper chromatography as demonstrated by either changing specific activity or $^{14}\text{C}/\text{H}$ peak ratios measured on the chromatograms (51-55). Several of the authors recognized and cautioned that the phenomenon must be taken into account for such studies as isotope dilution analysis of steroid secretion rates.

VI. Thin Layer Chromatography

The technique of thin layer chromatography (TLC) has only a few but certainly some of the most dramatic instances of isotopic fractionation yet observed. Perhaps the very first report of its occurrence on TLC was the example of [^{14}C] sodium formate, separated as a higher R_f spot on a basic silica gel system (56). TLC isotopic fractionation has also been reported for carbon-14 labeled dichlorobenzene isomers (57) as well as [1,2- ^3H] cholesterol (58), [2,4- ^3H] lithocholic acid (59), [^3H] Juvenile Hormone I (60), the calcium antagonist [^3H] VUF-4576 (61) and [^3H] methadone (62). Two studies on the TLC behavior of isotopically substituted analogues of imipramine have been described. Silica gel TLC of [benzene ring, N-methyl- ^3H] imipramine in basic solvent systems such as methanol:ammonia (200:3) caused separation of it as a lower R_f spot from the higher R_f unlabeled imipramine (63). However, isotopic fractionation was not observed in acidic silica gel TLC systems. Also, no isotopic fractionation was observed for [benzene ring- ^3H] imipramine, even in basic TLC systems. A photograph of the actual visualized TLC plates documented the separations observed in this study.

Our interest in tritiated radioligands for receptor binding studies prompted us also to study the TLC isotopic fractionation of [N-methyl- ^3H] imipramine (64). Our results completely paralleled those just described since we too observed pronounced isotopic fractionation between (lower R_f) [N-methyl- ^3H] imipramine and unlabeled imipramine in basic TLC systems but complete cochromatography between them in acidic TLC systems. Also, we saw no isotopic fractionation between [benzene ring- ^3H] imipramine or [N-methyl- ^{14}C] imipramine and unlabeled imipramine in any TLC system. In our case the measurement of TLC isotopic fractionation was exceptionally easy because of the ability after autoradiography to simply overlay the developed film upon the visualized TLC

plate. These results presented compelling evidence that the exclusive cause of isotopic fractionation for these compounds was deuterium or tritium substitution on the N-methyl groups. Therefore, although only a few examples of isotopic fractionation have been reported for TLC, it is important to be aware of its occurrence with the supportive role that this quick and convenient chromatographic method plays in the synthesis and characterization of labeled substances important to the life sciences.

VII. Gas Chromatography

With most of the aforementioned chromatography techniques, our discussion thus far of isotopic fractionation has been dominated by radioisotopes. Gas chromatography (GC) was a method where the property was first observed and widely studied for stable isotopes in general and deuterium in particular. Therefore it is only fitting to cover this isotope first. The subject was reviewed for selected small molecules almost thirty years ago by Van Hook (65).

The very first reported separation of any deuterated compound from its hydrogen analogue by GC would seem to be that of Wilzbach and Riesz (66). On a 4 meter didecyl phthalate column at 53°C with a helium flow rate of 45 mL/min, they were able to observe a partial separation of the earlier eluting perdeuterated cyclohexane from cyclohexane. Other workers using different GC conditions such as glass capillary columns (67), sandwiched capillary columns (68) and porous polymer beads (69) subsequently improved upon this same separation to almost baseline resolution. In these cases also, deuterated cyclohexane eluted faster than cyclohexane. Other reports of this separation (70, 71) as well as further examples of isotopic fractionation for other alkanes labeled with deuterium soon followed. They included 2,3-dimethylbutane (72), butane (73), ethane (74-78), ethylene dibromide (79) and alpha-1,2,3,4,5,6-hexachlorocyclohexane (80). The technique of recycle gas chromatography was exploited to enhance the separation factor in one of these investigations (73). Similar separations of a number of other deuterated alkanes have also been described (81-83). The isotopic fractionation of methane from its deuterated analogues has perhaps received the most attention (69, 73, 75, 77, 84-100). Some of these articles related the excellent separation of CH_4 and C^2H_4 with

the order of elution depending upon the GC conditions used. In one study (94) a marked difference in the separation between these isotopomers as a function of carrier gas on a Porapak S column was observed, and the degree of peak separation effected by each carrier gas was found to be in the order helium > argon > nitrogen > carbon dioxide. In several reports the reasonably clean separation of the four possible deuterated isotopomers of methane was achieved at low temperature. On a glass capillary column, the elution order was CH_4 , CH_3^1H , CH_2^2H_2 , CH^3H_3 and C^4H_4 (88). However, using graphon coated with 0.1% squalane, this elution order was reversed (77).

Besides saturated hydrocarbons, several other classes of deuterated compounds have demonstrated isotopic fractionation. Deuterium labeled olefins such as ethylene (69, 77, 101-112), propylene (101) and 2-butene (105) have been studied. In one of these reports (104) the use of a long silver nitrate-ethylene glycol column allowed nearly baseline separation of deuterated ethylenes differing by only a single deuterium. Additional modes of column complexation were operative in several other of these examples and in one of them (110) the use of a coordination compound, dicarbonylrhodium (1) 3-trifluoroacetylcamphorate, in squalane on a capillary column promoted the essentially complete resolution of all isotopomers of deuterated ethylene. The authors predicted that use of this technique would accomplish the separation of other deuterated olefins and that an enhanced separation could be anticipated for tritiated olefins. Other unsaturated deuterated hydrocarbons have also been found to exhibit isotopic fractionation (113).

Compounds labeled with deuterium such as benzene (69, 114-124), toluene (75, 116, 123-126) and xylenes (127-130) have also been examined. In one of these articles (115) the nature of the GC stationary phase was found to exert a profound effect on the degree of $\text{C}_6\text{H}_6/\text{C}_6^2\text{H}_6$ separation. Capillary columns coated with dinonyl phthalate, squalane and silicone oil afforded increasingly improved separations of the two compounds with perdeuterobenzene most often emerging as the earlier peak. In another study (116) almost complete separation of the isotopic pairs $\text{C}_6\text{H}_6/\text{C}_6^2\text{H}_6$ and $\text{C}_7\text{H}_8/\text{C}_7^2\text{H}_8$ was achieved via an open tubular thick layer graphitized carbon black column. One investigation (123) explored the relative influence that ring and side chain aliphatic deuteration would have in enhancing

peak separation and concluded that side chain labeling had more of an effect. The GC isotopic fractionation of other deuterated aromatics has been described (131, 132), and several reports about the separation of deuterated alkynes have also appeared. For deuterated acetylene (133) the order of elution on a Chromosorb P column was C_2H_4 , C_2H^3H and $C_2^2H_2$. However, for the isotopomers of deuterated 2-butyne the order of elution on a capillary column was reversed with the most deuterated species eluting first (134).

More polar compounds labeled with deuterium have also demonstrated isotopic fractionation on GC. These include acetone (69, 116, 135-141), acetonitrile (77), chloroform (135, 141), ethanol (135-138), methanol (77, 135-137), pyridine (116, 136, 137) and 2-butanol (142). In ref. 141 the use of cyclodextrins as host inclusion compounds for isotopomer separation was reported for the first time. It was demonstrated that not only was the cavity size a major factor in isotopic fractionation but also the cavity environment and cyclodextrin ring substituents were very influential. In the last study (142) the observation of the difference in NMR chemical shifts between deuterated and unlabeled alcohols with the NMR shift reagent $Eu(fod)_3$ inspired the authors to use it in effecting the GC separation. Further examples of GC isotopic fractionation for compounds labeled with deuterium include DMSO (143) and *N*-methylformamide and *N,N*-dimethylformamide (144). In the latter case it was noted that substitution with deuterium on the methyl group of the formamides lowered the GC retention time, but that its presence in the formal position increased retention time. GC isotopic fractionation of deuterated analogues has also been published for such ethers as tetrahydrofuran (145), where a cobalt (II) complex was employed, and di-2-butylether (146). The GC separations of several deuterated TMS carbohydrate derivatives (147-150) along with deuterated glucose and mannose (151) from unlabeled compounds have also been studied.

Other diverse deuterated compounds useful to life science investigators have manifested isotopic fractionation on GC including several examples of deuterated acids, amino acids or their TMS derivatives (152-156). It was found that deuterated TMS nucleoside analogues exhibited GC separation from their unlabeled counterparts (157). The deuterated analogues of uridine and cytidine were earlier

eluting but it was concluded that the degree of separation for each had more to do with structural considerations than label content. Other compounds labeled with deuterium such as TMS quinoxalins (158), caffeine and its metabolites (159, 160), iproniazid (161), aminopyrine (162), isopropylantipyrine (163), verapamil (164), desipramine (165, 166) and fentanyl (167) have all displayed some degree of GC isotopic fractionation.

Deuterated lipids and leukotrienes have also been the subject of GC isotopic fractionation studies. Deuterated fatty acid esters (168, 169) and metabolites of deuterated linoleic and linolenic acids (170) and deuterium labeled acetanilides of 6,9-octadecadiene (171) all displayed isotopic fractionation. Also, [$^2\text{H}_6$] valproic acid (172) and [$^2\text{H}_6$] 4-ketovalproic acid (173) separated on GC from the corresponding unlabeled compounds while a slight GC separation was noted between [$^2\text{H}_6$] LTB₄ and LTB₄ (174). Larger deuterated compounds like steroids have displayed GC isotopic fractionation from unlabeled counterparts too. [$^2\text{H}_6$] Testosterone (175) and [$^2\text{H}_6$] cholesterol (176) are such examples.

Although deuterated compounds have dominated the published instances of isotopic fractionation by GC, compounds labeled with tritium have also provided examples. By means of recycle GC, tritiated cyclobutane was separated as an earlier eluting peak from cyclobutane (73). Using a glass capillary column at low temperature the separation of CH_4 , CH_3^3H , CH_2^3H_2 , CH^3H_3 and C^3H_4 (increasing retention time) was reported (88). Curiously, the elution order was reversed using a charcoal column at low temperature (177). Other reports on the separation of tritiated methane isotopomers have appeared (178, 179) including one (180) which described the preparation and GC purification of C^3H_4 on a multi Curie scale. Other workers succeeded in the GC separation of several tritiated propane and butane isotopomers (181) and small reductions in the retention time of several compounds labeled with tritium including cyclohexane, cyclohexene, heptane and benzene compared to unlabeled compounds were noted (182). As with deuterated olefins, silver nitrate-ethylene glycol GC columns have promoted the separation of several tritiated olefins (183). A degree of GC isotopic fractionation has also been reported for larger tritiated molecules. Several tritiated lipid methyl esters have displayed some separation from their cold counterparts by GC (184, 185). Also,

monitoring the $^{14}\text{C}/^3\text{H}$ effluent ratio on the GC of certain tritiated steroids indicated that they were somewhat separated from their carbon-14 analogues (186).

Besides hydrogen isotopes, carbon isotopes have also demonstrated fractionation on GC. A small separation was noted for fatty acids labeled with carbon-14 (165). Also, a measure of GC separation has been observed for $^{13}\text{CO}/^{12}\text{CO}$ (187-189) and $^{12}\text{CO}_2/^{13}\text{CO}_2$ (190, 191), $^{12}\text{CH}_4/^{13}\text{CH}_4$ (192, 193) and $^{12}\text{CF}_4/^{13}\text{CF}_4$ (194). The investigation of isotopic fractionation for $^{12}\text{CH}_4/^{13}\text{CH}_4$ in ref. 193 is intriguing because it was prompted by the disparate ratios of these isotopomers in samples taken at various locations in a southern Italian gas field. The authors used Bentonite as a GC solid support to mimic what they propose was geological isotopic fractionation. Isotopic fractionation by GC for fatty acid methyl esters and other compounds labeled with carbon-13 has also been observed (195, 196). The GC isotopic fractionation of $^3\text{HCH}_4$ from methane facilitated the improvement of its specific activity (197, 198), and a degree of GC isotopic fractionation was also reported for some nitrogen-15 labeled compounds (199).

VIII. High Pressure Liquid Chromatography

The most recent chromatographic technique to provide examples of isotopic fractionation is high pressure liquid chromatography (HPLC). In this area, reports of the phenomenon emerged almost simultaneously for both tritium and deuterium labeled compounds. With tritium, among the earliest examples reported were the reverse phase HPLC isotopic fractionation of the polycyclic aromatics [^3H] 7,12-dimethylbenz[a]anthracene, [^3H] benzo[a]pyrene and analogues (200-202). In these instances the tritiated material often eluted earlier than the cold standard. The HPLC of arachidonic acid metabolites on silver ion loaded columns also exhibited isotopic fractionation, with the separation of the earlier eluting unlabeled deuterated or carbon-14 compounds from the later eluting tritiated analogues (203-205). The authors proposed a unique and compelling explanation for the observation (see Section IX). Further reports concerning the HPLC isotopic fractionation of [^3H] arachidonic acid and other eicosanoids labeled with tritium followed (206-210), with several of the authors reflecting on the implications of the

phenomenon for biological assays. In the last reference Do and coworkers summarized over a decade of observations made in their laboratory on such separations for both normal and reverse phase HPLC with a variety of immobile phases. On reverse phase HPLC several tritiated steroids (55, 211) and on both normal and reverse phase HPLC a number of tritiated Vitamin D analogues displayed isotopic fractionation (212-216). Several of the authors noted that the degree of separation was influenced by tritium location. Worth (216) also speculated that the pronounced isotopic fractionation could be exploited to elevate the specific activity of tritiated Vitamin D metabolites.

Tritiated nucleosides and related compounds have also been studied. [^3H] Thymine (217) and [^3H] thymidine (218) analogues as well as [^3H] 2'-deoxyguanosine (219) were reported to separate on HPLC from unlabeled standards. The Kudelin group in St. Petersburg has employed isotopic fractionation on preparative HPLC to enhance the specific activity of tritiated nucleosides (220). In this account, specific activities were significantly increased with selective fraction collection of earlier eluting labeled compounds. [^3H] Dopamine (221) has also exhibited isotopic fractionation on reverse phase HPLC. This thorough study explored the influence of tritium location on separation and would appear to be one of the earliest accounts of the phenomenon for catecholamines. The drug substances [^3H] bepridil (222), [^3H] imipramine, [^3H] desipramine (223) and [^3H] N-0437 (224), and the macrocycle [^3H] tetrahydroechinocandin B (225) have also displayed HPLC peak fractionation. In the first two examples the authors noted a marked effect on peak separation in conjunction with the proximity of tritium to nitrogen. In contrast, the last example, initially disclosed at an October 1990 Merck hosted International Isotope Society meeting, is remarkable for the degree of separation provided by tritium in an extended alkyl chain location of the macrocycle remote from any heteroatom.

Several [N-methyl- ^3H] radioligands have also afforded interesting cases of HPLC isotopic fractionation. On normal phase HPLC eluted with dichloromethane:methanol (98:2), we found that the elution order of (+-) mianserin analogues were unlabeled (+-) mianserin, (+-)-[N-methyl- ^3H] mianserin and finally (+-)-[N-methyl- ^3H] mianserin (226). The simultaneous use of both UV detection and liquid scintillation flow monitoring facilitated our measurement of isotopic

fractionation in this study. On reverse phase HPLC both [N-methyl- ^3H] chlorpromazine (227) and [N-methyl- ^3H] SKF (R) 83566 (228) demonstrated analogous HPLC separation. Interestingly, the retention time of the former radioligand was longer than the unlabeled standard while that of the latter was shorter than unlabeled standard on reverse phase HPLC. Also in the earlier study, no isotopic fractionation was reported for [benzene ring- ^3H] chlorpromazine and the degree of isotopic fractionation for [N-methyl- ^3H] chlorpromazine was significantly dependent upon pH.

For deuterated compounds the first example of HPLC isotopic fractionation recorded would seem to be the separation of long chain perdeutero carboxylic acids (earlier eluting) from unlabeled standards on reverse phase by Thornton and Tanaka (229, 230). Baseline peak separation was noted for palmitic acid and perdeuteropalmitic acid prompting the authors to suggest the significance of their findings for possible preparative scale utility. Motivation for this study of the isotope effect during "hydrophobic binding" was its possible role as a model for similar interactions in biomembranes. [^3H] Methyl palmitate was separated from unlabeled standard employing a recycle technique on silver nitrate impregnated silica gel HPLC (231). This and similar methodology was later applied to other deuterated fatty acids (232, 233).

HPLC isotopic fractionation of monodeuterobenzene (234) as well as perdeuterobenzene (235-242), perdeuterotoluene (235, 238, 242, 243) and ethyl benzene (244) have been described. In most cases the deuterated compound eluted earlier than the unlabeled one. In one of these reports (239) it was concluded that deuterated water was superior to water for the HPLC separation of deuterated aromatics from unlabeled compounds, enhancing the separation by as much as 30%. Other deuterated aromatic compounds also have been investigated including [^3H] benzoic acid (245, 246). In the first study it was apparent that the position of deuterium labeling had a significant effect on the degree of fractionation observed, with ortho substitution being far less influential for separation than meta or para substitution. The author was inclined to propose a steric rather than electronic explanation for the observation. In the case of several aromatic aldehydes labeled

with deuterium on the aldehyde carbonyl, Yu and coworkers have described their HPLC separation from unlabeled compounds (247-249).

Other deuterated aromatic compounds reported to exhibit isotopic fractionation include [^2H] naphthalene 2-sulfonic acid (250), several deuterated polycyclic aromatics (251), [^2H] dopamine (252), [^2H] epinephrine and [^2H] norepinephrine (253) and [^2H] indole-3-acetic acid (254, 255). Several deuterated steroids (208, 256) and eicosanoids (204, 205, 257) have shown a degree of HPLC isotopic fractionation. Also the vitamin A metabolic precursor B-carotene, when deuterated, was found to separate with baseline resolution from unlabeled compound on reverse phase HPLC (258, 259), and in both reports the labeled compound eluted earlier. In the second study the authors showcased the results as an opportunity for other investigators without access to a mass spectrometer to study the dynamics of absorption and metabolism of [^2H] B-carotene in humans.

Deuterated drug type compounds have provided further examples of HPLC isotopic fractionation. These include [^2H] N-0437 glucuronide (224) several deuterated caffeine (260), [^2H] methadone (261), and some deuterated benzodiazepines and related compounds (236, 262-264). The effect of pH was noted as influential in some of these separations (262, 263). Also investigated were the tricyclic [N-methyl- ^2H] imipramine (265) and the tetracyclic [^2H] mianserin (266, 267). In this latter example it was clear that only deuterium substitution on the piperazine ring of mianserin would promote the observed separation. The Organon group also investigated the related compound [^2H] Org GC-94 (268) which on normal phase eluted later than the unlabeled compound. In this case the more acidic the eluent, the better was the observed peak separation. Other examples of related HPLC isotopic fractionation include [^2H] carbamazepine (269), [^2H] CCNU analogues (270) and [^2H] roxatidine acetate metabolites (271) and [^2H] bepridil (222).

Various other deuterated compounds have also been reported to display isotopic fractionation including [^2H] tetrahydroechinocandin B (225) and several [^2H] gibberellins (272). In this latter case the labeled gibberellins eluted slightly ahead of the endogenous substances on reverse phase HPLC. Even the pigment chlorophyll (one of the earliest substances to be studied chromatographically by

Tswett) when deuterated, has demonstrated some striking reverse phase HPLC isotopic fractionation (273-275). Using the mobile phase water:methanol:acetonitrile:tetrahydrofuran (5:28:38:23) with a flow rate of 1 mL/min, Baweja reported that [^2H] chlorophyll *a* eluted two minutes ahead of the unlabeled compound (273). Reverse phase HPLC also accomplished the separation of [N-methyl- ^2H] FTC-methylamine (276) as well as some perdeuterated diglucosyldiacylglycerols (277) from their unlabeled counterparts.

Other isotopes besides hydrogen have provided examples of HPLC fractionation. Both [^{14}C] trioleoylglycerol (278) and [^{14}C] leucine (279) appeared to separate from unlabeled standards. Further examples are [^{13}C] theophylline metabolites (280), [^{13}C] leucine (281), the antiviral compound [^{13}C] LY-217,896 (282), [^{13}C] decanal (283), some organic acids labeled with carbon-13 (284) and [^{13}C] methyl palmitate (285). Tanaka and coworkers have elegantly explored the isotopic fractionation of both nitrogen-15 and oxygen-18 labeled compounds. Although not completely resolved, significant HPLC separations of [^{15}N] aniline (286, 287), and [^{15}N] N-methylaniline and [^{15}N] N,N-dimethylaniline (287, 288). Others have reported similar HPLC isotopic fractionation of [^{15}N] chlorophyll (289). Tanaka has also reported the HPLC isotopic fractionation of the several isotopomers of [^{18}O] benzoic acid (290-292), [^{18}O] 4-chlorobenzoic acid (292) and [^{18}O] 4-nitrophenol (287, 292).

Perhaps the most intriguing aspect of isotopic fractionation for HPLC or any other chromatographic method has been very recently reported; namely, chromatographic separation based on the chirality conferred by isotopic content as initially reported by the Tanaka group (293-295). Indeed, in his first paper (293) Tanaka cited the fact that prior claims of diastereomeric separation based on isotopic influence have been challenged (296). In this demonstration of chiral isotopic fractionation, diastereomers of [phenyl- ^2H] methyl 3-diphenylglycidate were separated on reverse phase HPLC as three peaks with the perdeuterated species eluting first. The center peak containing the two diastereomeric pairs created by single phenyl ring deuteration was separately caught and injected onto a chiral HPLC column whereby enantiomer separation was observed. Another equally compelling example of this phenomenon was reported recently by Pirkle (297).

disclosing that deuterated enantiomers of a pivalamide were separated in a chiral supercritical fluid HPLC experiment.

IX. Causes of Isotopic Fractionation

Although a great deal of effort has been expended in documenting various instances of isotopic fractionation, less time has been spent in explaining its mechanism. Indeed, after considering all the published work on the subject, it appears that a number of factors, varying in importance and each additive or subtractive in contributing to peak separation, may be operative. However, when considered collectively, these factors should adequately explain the elution order of substances differing only in isotopic content.

Mass:

In most cases the mass difference between isotopically labeled and unlabeled compounds alone has been dismissed as playing any significant role in isotopic fractionation. Isotopic substitution in many of the compounds reviewed here alter their molecular weight by only a few percent at best. However, the GC elution order of $^{12}\text{CH}_4$, $^{13}\text{CH}_4$, and $^{14}\text{CH}_4$ was consistent with their increasing mass differences (88).

Steric Considerations:

One would think that steric effects produced by the isotope itself would rarely be invoked as a cause of isotopic fractionation. However, a persuasive explanation advanced for the isotopic fractionation of some deuterated and tritiated compounds is a steric one; namely, the lowered zero point vibrational frequency and resulting decrease in the Van-der-Waals radius of the series H , ^2H , ^3H (298), and the increasingly shorter carbon-hydrogen isotope bond length in the series C-H , $\text{C-}^2\text{H}$ and $\text{C-}^3\text{H}$. For instance: 1) On a silver nitrate-ethylene glycol GC column, the deuterated isotopomers of ethylene (101, 104) and propylene (101) can be separated. 2) On a silver nitrate-silica gel column, [9, 10, 12, 13- ^3H] methyl linoleate migrated more slowly than the unlabeled standard (23). 3) On a silver nitrate impregnated normal HPLC column, [1- ^{14}C] PGE₂ eluted faster than [5, 6, 8, 11, 12, 14, 15- ^3H] PGE₂ (203). An explanation for all three of the foregoing separations is that the

decreasing C-H, C-³H and C-²H bond lengths allowed increasing interaction between the complexing silver ions of the solid support and the olefins of the isotopomers in question. It has been observed that the stability of silver ion-olefin complexes increased when the hydrogen atoms were replaced by deuterium (299). In this same study it was also noted that such silver ion-olefin complexes were more stable when deuterium was actually attached to the olefin bond rather than merely adjacent to it. Therefore, it can be argued that the progressive substitution of deuterium or tritium for hydrogen would retard to a greater degree the migration of an olefin on a silver ion loaded column. A similar explanation may be operative for the inclusion mechanism based separation of deuterated compounds in ref. 26 and the HPLC separation of deuterated benzoic acid isotopomers (245).

Vapor Pressure:

The vapor pressure of a labeled and unlabeled compound can differ (300-303). For instance, the vapor pressure of a deuterated compound is generally higher than that of the corresponding unlabeled compound (304). Certainly, this circumstance could be a factor influencing isotopic fractionation by GC.

Polarity:

The alteration of an atom's polarity by an adjacent isotope has been cited as the cause of some of the most intriguing instances of isotopic fractionation. From the work of Robertson (305, 306), it would seem that the substitution of deuterium or tritium on a carbon atom bearing a nitrogen would cause the nitrogen to be more basic than its unlabeled counterpart. During silica gel chromatography, the amine would more strongly deprotonate the weakly acidic Si-OH groups and thus retard the elution of the labeled compound relative to the unlabeled one. Obviously, this effect would be more pronounced in neutral or basic eluents, but in systems acidic enough to protonate all amines, such isotopic fractionation would normally be minimal. Polarity has also been suggested for the observed HPLC isotopic fractionation of aldehydes substituted at their carbonyl with deuterium, potentially altering their hydrophobic interaction with the reverse phase chromatographic system (247-249).

X. Implications

Having reviewed the many examples and probable causes of isotopic fractionation during the past several decades it is appropriate to briefly consider its implications. Clearly isotopic fractionation when considered over the entire experience of labeled compound chromatography is still a rare but certainly precedented observation and should only be invoked cautiously. If it is operative and not taken into account then significant errors can corrupt the measurements made with labeled compounds such as specific activity (33), various assays (209, 211) and mass spectrometry techniques (307). Awareness of it can prompt the use of an alternate label that does not display the phenomenon. Furthermore, isotopic fractionation has been exploited to facilitate various measurements including physical adsorption (91, 92), the dynamics of complexation (110), GC-MS analysis (154) and partition coefficient determination (241). It has also been used to substitute for more costly analyses like GC-MS (259) and provide models for such systems as biomembranes (229, 230).

References

1. Klein P. D. - *Adv. In Chrom.* 3: 3 (1966)
2. Sera S., Ikeda H. and Goromaru T. - *Fukuyama Daigaku Yakugakubu Kenkyu Nenpo* 13: 1 (1995)
3. Leseticky L. - *Radioisotopy* 26: 113 (1985)
4. Matucha M. and Smolkova E. - *J. Chrom.* 127: 163 (1976)
5. Cram S. P. - *Adv. In Chrom.* 9: 243 (1970)
6. Eiler C. N. - Abstracts, Sixth Intern. Symp. Synth. Applic. Isotop. Label. Compd., Philadelphia, Penn., #PB-4 (1997)
7. Marshall L. M. and Cook R. E. - *J. Amer. Chem. Soc.* 84: 2647 (1962)
8. Marshall L. M. and Magee D. - *J. Chrom.* 15: 97 (1964)
9. Kirdani R. Y. and Priore R. L. - *Anal. Biochem.* 35: 13 (1970)
10. Van Dyken A. R. - Abstracts 128th Natl. Meet. Amer. Chem. Soc., Minneapolis, pg. 28-O (1955)
11. Brown W. G., Kaplan L., Van Dyken A. R. and Wiltzbach K. E. - *Proc. Intern. Conf. Peaceful Uses of Atomic Energy* 15: 16 (1956)
12. Klein P. D. - *Atomlight* 41: 1 (1964)

13. Klein P. D., Simborg D. W. and Szczepanik P. A. - *Pure Appl. Chem.* **8**: 357 (1964)
14. Klein P. D. - *Adv. Tracer Method.* **2**: 145 (1965)
15. Cejka V. and Venneman E. M. - *Clin. Chim. Acta* **11**: 188 (1965)
16. Cejka V. and Venneman E. M. - *Adv. Tracer Method.* **3**: 173 (1966)
17. Cejka V. and Venneman E. M. - *Atomlight* **55**: 1 (1966)
18. Cejka V., Venneman E. M., Bosch N. B. -V. D. and Klein P. D. - *J. Chrom.* **22**: 308 (1966)
19. Klein P. D., Cejka V. and Szczepanik P. A. - *Adv. Tracer Method.* **4**: 105 (1968)
20. Paliokas A. M. and Schroepfer G. J. - *J. Biol. Chem.* **243**: 453 (1968)
21. Xing S., Zheng S. and Cekan S. Z. - *Clin. Chem.* **27**: 600 (1981)
22. Bollenbacher W. E., Galbraith M. N., Gilbert L. I. and Horn D. H. S. - *Steroids* **29**: 47 (1977)
23. Sgoutas D. S. and Kummerow F. A. - *J. Chrom.* **16**: 448 (1964)
24. Sakurai T., Ichimiya H., Miyazaki H. and Nakayama F. - *J. Chrom.* **571**: 1 (1991)
25. Strain H. H., Thomas M. R., Crespi H. L. and Katz J. J. - *Biochim. Biophys. Acta* **52**: 517 (1961)
26. Ofodile S. E. and Smith N. O. - *Anal. Chem.* **53**: 904 (1981)
27. Piez K. A. and Eagle H. - *Science* **122**: 968 (1955)
28. Piez K. A. and Eagle H. - *J. Amer. Chem. Soc.* **78**: 5284 (1956)
29. Piez K. A. - *Anal. Biochem.* **4**: 444 (1962)
30. Bellobono I. R. and Galazzi G. - *Int. J. Appl. Rad. Isot.* **17**: 257 (1966)
31. Bellobono I. R. - *Rend. Ist. Lombardo Sci. Lettere* **A99**: 336 (1965)
32. Bellobono I. R. - *J. Chrom.* **34**: 515 (1968)
33. Gaitonde M. K. and Nixey R. W. K. - *Anal. Biochem.* **50**: 416 (1972)
34. Davidson C. N., Mann C. K. and Sheline R. K. - *J. Phys. Chem.* **67**: 1519 (1963)
35. Gottschling H. and Freese E. - *Nature* **196**: 829 (1962)
36. Klein P. D. and Szczepanik P. A. - *Anal. Chem.* **39**: 1276 (1967)
37. Klein P. D. and Szczepanik P. A. - *J. Chrom.* **42**: 493 (1969)
38. Shoaf C. R., Caplow M. and Heizer W. D. - *Anal. Biochem.* **96**: 12 (1979)
39. Shoaf C. R., Heizer W. D. and Caplow M. - *Carbohydr. Res.* **103**: 195 (1982)
40. Connor M. J., Blair J. A. and Said H. - *Nature* **287**: 253 (1980)
41. Forbush B. and Hoffman J. F. - *Biochem.* **18**: 2308 (1979)
42. Hirschberg K., Krumbiegel P. and Faust H. - *Isotopenpraxis* **17**: 178 (1981)
43. Rohrer J. S. and Olechno J. D. - *Anal. Chem.* **64**: 914 (1992)
44. Giovannelli J., Mudd S. H. and Datko A. H. - *J. Biol. Chem.* **253**: 5665 (1978)
45. Bushey M. M. and Jorgenson J. W. - *Anal. Chem.* **61**: 491 (1989)
46. Bushey M. M. and Jorgenson J. W. - *J. Microcolumn Sep.* **1**: 125 (1989)
47. Chiari M., Nesi M., Ottolina G. and Righetti P. G. - *J. Chrom. A* **680**: 571 (1994)

48. Terabe S., Yashima T., Tanaka N. and Araki M. - *Anal. Chem.* **60**: 1673 (1988)
49. Blake M. I., Crespi H. L., Mohan V. and Katz J. J. - *J. Pharm. Sci.* **50**: 425 (1961)
50. Koch H. J. and Stuart R. S. - *Carbohydr. Res.* **64**: 127 (1978)
51. Jensen E. V. and Jacobson H. I. - *Recent Prog. Hormone Res.* **18**: 387 (1962)
52. Laragh J. H., Sealey J. E. and Klein P. D. - *Proc. Symp. Radiochem. Methods Anal.* **2**: 353 (1965)
53. Benraad T. J. and Kloppenborg P. W. C. - *Clin. Chim. Acta* **12**: 565 (1965)
54. Gold N. I. and Crigler J. F. - *J. Clin. Endocrin.* **26**: 133 (1966)
55. Hollandt H., Kraas E. and Knuppen R. - *Fresenius Z. Anal. Chem.* **311**: 431 (1982)
56. Viswanathan K. V. - *Int. J. Appl. Radiat. Isot.* **16**: 60 (1965)
57. Ajami A. M. - *Trans. Amer. Nuc. Soc.* **55**: 33 (1987)
58. Wood P. D. S., Myers D., Lee Y.-L., Shioda R. and Kinsell L. W. - *J. Lipid Res.* **8**: 406 (1967)
59. Hofmann A. F., Szczepanik P. A. and Klein P. D. - *J. Lipid Res.* **9**: 707 (1968)
60. Hamnett A. F. and Pratt G. E. - *J. Chrom.* **158**: 387 (1978)
61. Caldirola P. and Timmerman H. - *J. Label. Compd. Radiopharm.* **31**: 987 (1992)
62. Gerardy B. M., Poupaert J. H., Vandervorst D., Dumont P., Declercq J.-P., Van Meerssche M. and Portoghese P. S. - *J. Label. Compd. Radiopharm.* **22**: 5 (1985)
63. D'A. Heck H., Simon R. L. and Anbar M. - *J. Chrom.* **133**: 281 (1977)
64. Filer C. N., Ahern D. G., Fazio R. and Johnson E. - *Abstracts, Second Intern. Symp. Synth. Applic. Isotop. Label. Compd., Kansas City, Mo., #PB-4* (1985)
65. Van Hook W. A. - *Adv. Chem. Ser.* **89**: 99 (1969)
66. Wilzbach K. E. and Riesz P. - *Science* **126**: 748 (1957)
67. Bruner F. A. and Cartoni G. P. - *Anal. Chem.* **36**: 1522 (1964)
68. Liberti A., Nota G. and Goretti G. - *J. Chrom.* **38**: 282 (1968)
69. Possanzini M., Pela A., Liberti A. and Cartoni G. P. - *J. Chrom.* **38**: 492 (1968)
70. Bosshard C., Piringier O. and Gaumann T. - *Helv. Chim. Acta* **54**: 1059 (1971)
71. Sterkhov N. V., Ravikovich V. M., Litvin E. F. and Chizhkov V. P. - *Zh. Fiz. Khim.* **57**: 2897 (1983)
72. Falconer W. E. and Cvetanovic R. J. - *Anal. Chem.* **34**: 1064 (1962)
73. Root J. W., Lee E. K. C. and Rowland F. S. - *Science* **143**: 676 (1964)
74. Van Hook W. A. and Kelly M. E. - *Anal. Chem.* **37**: 508 (1965)
75. Bruner F., Cartoni G. P. and Liberti A. - *Anal. Chem.* **38**: 298 (1966)
76. Van Hook W. A. and Phillips J. T. - *J. Phys. Chem.* **70**: 1515 (1966)
77. DiCorcia A., Fritz D. and Bruner F. - *J. Chrom.* **53**: 135 (1970)

78. de Zeeuw J., de Nijs R. C. M. and Henrich L. T. - *J. Chrom. Sci.* **25**: 71 (1987).
79. Keough T., Strife R. J., Rodriguez P. A. and Sanders R. A. - *J. Chrom.* **312**: 450 (1984).
80. Vetter W. and Luckas B. - *J. High Res. Chrom.* **18**: 643 (1995).
81. Schömburg G. and Henneberg D. - *Chromatographia* **1**: 23 (1968).
82. Gaumann T. and Bonzo R. - *Helv. Chim. Acta* **56**: 1165 (1973).
83. Matucha M. - *Chromatographia* **27**: 552 (1989).
84. Bruner F. and Cartoni G. P. - *J. Chrom.* **18**: 390 (1965).
85. Van Hook W. A. - *J. Phys. Chem.* **71**: 3270 (1967).
86. Czubryt J. J. and Gesser H. D. - *J. Gas Chrom.* **6**: 41 (1968).
87. Alberini G., Bruner F. and Devitofrancesco G. - *Anal. Chem.* **41**: 1940 (1969).
88. Bruner F., Cartoni G. P. and Possanzini M. - *Anal. Chem.* **41**: 1122 (1969).
89. DiCorcia A. and Bruner F. - *J. Chrom.* **49**: 139 (1970).
90. Norton P. R. - *J. Chrom. Sci.* **8**: 411 (1970).
91. DiCorcia A. and Liberti A. - *Trans. Farad. Soc.* **66**: 967 (1970).
92. Phillips J. T. and Van Hook W. A. - *J. Phys. Chem.* **71**: 3276 (1967).
93. Sakodyskii K. I. and Sevast'yanova A. K. - *Gaz. Khromatogr.* **10**: 70 (1969).
94. Czubryt J. J., Gesser H. D. and Bock E. - *J. Chrom.* **53**: 439 (1970).
95. Bruner F., Canulli C., DiCorcia A. and Liberti A. - *Nature* **231**: 175 (1971).
96. Bruner F., Canulli C., Liberti A. and Marchetti M. - *Gazz. Chim. Ital.* **103**: 877 (1973).
97. Kiselev A. V., Khudyakov V. L. and Yashin Y. I. - *Zh. Fiz. Khim.* **48**: 448 (1974).
98. Lukac S. - *J. Chrom.* **166**: 287 (1978).
99. Ott D. G., Kerr V. N., Sanchez T. G. and Whaley T. W. - *J. Label. Compd. Radiopharm.* **17**: 255 (1979).
100. Mohnke M. and Heybey J. - *J. Chrom.* **471**: 37 (1989).
101. Cvctanovic R. J., Duncan F. J. and Falconer W. E. - *Can. J. Chem.* **41**: 2095 (1963).
102. Dubrin J., MacKay C. and Wolfgang R. - *J. Amer. Chem. Soc.* **86**: 959 (1964).
103. Ache H. J. and Wolf A. P. - *J. Amer. Chem. Soc.* **88**: 888 (1966).
104. Atkinson J. G., Russell A. A. and Stuart R. S. - *Can. J. Chem.* **45**: 1963 (1967).
105. Wasik S. P. and Tsang W. - *J. Phys. Chem.* **74**: 2970 (1970).
106. Ache H. J. and Wolf A. P. - *Z. Anal. Chem.* **230**: 19 (1967).
107. Cartoni G. - *Corso Semin. Chim.* **2**: 51 (1968).
108. Schurig V. and Gil-av E. - *J. Chem. Soc. Chem. Commun.* **650** (1971).
109. Schurig V., Chang R. C., Zlatkis A., Gil-av E. and Mikes F. - *Chromatographia* **6**: 223 (1973).
110. Schurig V. - *Angew. Chem. Int. Ed.* **15**: 304 (1976).
111. Schurig V. - *Chromatographia* **13**: 263 (1980).
112. Youn D. Y., Hong K. B., Jung K.-H., Kim D. and Kim K.-R. - *J. Chrom.* **607**: 69 (1992).

113. Schomburg G. and Husmann H. - *Chromatographia* **8**: 517 (1975)
114. Bruner F. and Cartoni G. P. - *J. Chrom.* **10**: 396 (1963)
115. Liberti A., Cartoni G. P. and Bruner F. - *J. Chrom.* **12**: 8 (1963)
116. Goretti G. C., Liberti A. and Nota G. - *J. Chrom.* **34**: 96 (1968)
117. Sakodinskii K. I., Khudyakov V. L. and Yashin Y. I. - *Isotopenpraxis* **4**: 278 (1968)
118. Van Kemenade A. W. C. and Groenendijk H. - *Chromatographia* **2**: 148 (1969)
119. Wasik S. P. and Tsang W. - *Anal. Chem.* **42**: 1649 (1970)
120. Tsang W. and Wasik S. P. - *J. Chrom. Sci.* **9**: 567 (1971)
121. Chizhkov V. P. and Sinitsyna L. A. - *Zh. Fiz. Khim.* **48**: 142 (1974)
122. Chizhkov V. P., Pavlov S. S., Sterkhov N. V., Ravikovich V. M., Litvin E. F. and Varyvovich E. A. - *Talanta* **34**: 227 (1987)
123. Shi B. and Davis B. H. - *J. Chrom. A* **654**: 319 (1993)
124. Macnaughtan D. and Rogers L. B. - *Sep. Sci.* **7**: 307 (1972)
125. Sakodinskii K. I., Cartoni G. P. and Pela A. - *Zh. Fiz. Khim.* **40**: 2887 (1966)
126. Zabokritskii M. P., Chizhkov V. P. and Rudenko B. A. - *J. High Res. Chrom. Chrom. Commun.* **8**: 170 (1985)
127. Kwa T. L. and Boelhouwer C. - *J. Chrom.* **52**: 21 (1970)
128. Bermejo J., Blanco C. G. and Guillen M. D. - *J. Chrom.* **351**: 425 (1986)
129. Childers J. W., Wilson N. K. and Barbour R. K. - *Anal. Chem.* **64**: 292 (1992)
130. Eganhouse R. P., Dorsey T. F., Phinney C. S. and Westcott A. M. - *J. Chrom.* **628**: 81 (1993)
131. Mercer J. R. and Wiebe L. I. - *J. Label. Compd. Radiopharm.* **14**: 445 (1978)
132. Durden D. A. and Boulton A. A. - *J. Neurochem.* **36**: 129 (1981)
133. Van Hook W. A. and Phillips J. T. - *J. Chrom.* **30**: 211 (1967)
134. Saussey J., Lavalley J. C., Janin A. and Vallet A. - *J. Label. Compd. Radiopharm.* **13**: 417 (1977)
135. Cartoni G. P., Liberti A. and Pela A. - *Anal. Chem.* **39**: 1618 (1967)
136. Sakodinskii K. I., Le L.-C. and Alikhanov P. P. - *J. Chrom.* **77**: 21 (1973)
137. Le L.-C. and Sakodinskii K. I. - *Zh. Fiz. Khim.* **46**: 1510 (1972)
138. Chizhkov V. P. and Sinitsyna L. A. - *Zh. Anal. Khim.* **29**: 600 (1974)
139. Chizhkov V. P. and Sinitsyna L. A. - *J. Chrom.* **104**: 327 (1975)
140. Zabokritskii M. P., Rudenko B. A. and Chizhkov V. P. - *Izv. Akad. Nauk SSSR Ser. Khim.* **7**: 1627 (1977)
141. Andrews A. R. J., Wu Z. and Zlatkis A. - *Chromatographia* **34**: 457 (1992)
142. Brooks J. J. and Sievers R. E. - *J. Chrom. Sci.* **11**: 303 (1973)
143. Liberti A. and Zoccolillo L. - *J. Chrom.* **49**: 18 (1970)
144. Mraz J., Jheeta P., Gescher A. and Threadgill M. D. - *J. Chrom.* **641**: 194 (1993)
145. Schurig V. and Wistuba D. - *Angew. Chem. Int. Ed.* **22**: 772 (1983)
146. Shi B., Keogh R. A. and Davis B. H. - *J. Chrom. A* **678**: 97 (1994)

147. Bentley R., Saha N. C. and Sweeley C. C. - *Anal. Chem.* **37**: 1118 (1965)
148. Sweeley C. C., Elliott W. H., Fries I. and Ryhage R. - *Anal. Chem.* **38**: 1549 (1966)
149. Saha N. C. and Sweeley C. C. - *Anal. Chem.* **40**: 1628 (1968)
150. Waller G. R., Sastry S. D. and Kinneberg K. - *J. Chrom. Sci.* **7**: 577 (1969)
151. Mercea V. - AEC Accession No. 26745, Rept. No. IFA-IS-34
152. Meier-Augenstein W., Hoffmann G. F., Holmes B., Jones J. L., Nyhan W. L. and Sweetman L. - *J. Chrom.* **615**: 127 (1993)
153. VandenHeuvel W. J. A., Smith J. L. and Cohen J. S. - *J. Chrom. Sci.* **8**: 567 (1970)
154. VandenHeuvel W. J. A., Smith J. L., Putter I. and Cohen J. S. - *J. Chrom.* **50**: 405 (1970)
155. Zolotarev Y. A., Zaitsev D. A., Lubnin M. Y., Tatur V. Y. and Myasoyedov N. P. - *Appl. Radiat. Isot.* **39**: 619 (1988)
156. Zee T., Stellaard F. and Jakobs C. - *J. Chrom.* **574**: 335 (1992)
157. Hattox S. E. and McCloskey J. A. - *Anal. Chem.* **46**: 1378 (1974)
158. Langenbeck U., Mohring H.-U. and Dieckmann K.-P. - *J. Chrom.* **115**: 65 (1975)
159. Cherrah Y., Falconnet J. B., Desage M., Brazier J. L., Zini R. and Tillement J. P. - *Biomed. Env. Mass. Spectrom.* **14**: 653 (1987)
160. Bencheikroun Y., Dautraix S., Desage M. and Brazier J. L. - *J. Chrom. B* **688**: 245 (1997)
161. Goromaru T., Ikejiri H., Hashimoto K. and Matsuki Y. - *Yakugaku Zasshi* **111**: 612 (1991)
162. Goromaru T., Maeda H., Matsuki Y. and Kiwada H. - *Radioisotopes* **42**: 511 (1993)
163. Goromaru T. and Maeda H. - *Biol. Pharm. Bull.* **17**: 1635 (1994)
164. Hoffman D. J. and Higgins J. - *J. Chrom.* **374**: 170 (1986)
165. Matucha M., Jockisch W., Verner P. and Anders G. - *J. Chrom.* **588**: 251 (1991)
166. Matucha M. - Abstracts, Fifth Intern. Symp. Synth. Applic. Isotop. Label. Compd., Strasbourg, France, PB008 (1994)
167. Sera S. and Goromaru T. - *Radioisotopes* **46**: 885 (1997)
168. Wendt G. and McCloskey J. A. - *Biochemistry* **9**: 4854 (1970)
169. McCloskey J. A., Lawson A. M. and Leemans F. A. J. M. - *J. Chem. Soc. Chem. Commun.* **285** (1967)
170. Pawlosky R. J., Sprecher H. W. and Salen N. - *J. Lipid Res.* **33**: 1711 (1992)
171. Leemans F. A. J. M. and McCloskey J. A. - *J. Amer. Oil Chem. Soc.* **44**: 11 (1967)
172. Hoffman D. J. and Porter W. R. - *J. Chrom.* **276**: 301 (1983)
173. Yu D., Gordon J. D., Zheng J., Panesar S. K., Riggs K. W., Rurak D. W. and Abbott F. S. - *J. Chrom. B* **666**: 269 (1995)

174. Dawson M., Vine J. H., Forrest M. J., McGee C. M., Brooks P. M. and Watson T. R. - *J. Label. Compd. Radiopharm.* **24**: 291 (1987)
175. Tcholakian R. K., Steinberger A. and Newaz S. N. - *J. Steroid Biochem.* **26**: 619 (1987)
176. Wasilchuk B. A., Le Quesne P. W. and Vouras P. - *Anal. Chem.* **64**: 1077 (1992)
177. Gant P. L. and Yang K. - *J. Amer. Chem. Soc.* **86**: 5063 (1964)
178. Ciranni G. and Guarino A. - *J. Label. Compd. Radiopharm.* **2**: 198 (1966)
179. Leonov V. V., Sakodinskii K. I., Sinotova E. N. and Korsakov M. V. - *Zh. Fiz. Khim.* **48**: 2343 (1974)
180. Cacace F. and Schuller M. - *J. Label. Compd. Radiopharm.* **11**: 313 (1975)
181. Bruner F., Ciccioli P. and DiCorcia A. - *Anal. Chem.* **44**: 894 (1972)
182. Gordon B. E., Olivos J. W., Erwin W. R. and Lemmon R. M. - *Int. J. Appl. Radiat. Isot.* **33**: 721 (1982)
183. Lee E. K. C. and Rowland F. S. - *Anal. Chem.* **36**: 2181 (1964)
184. Sgoutas D. S. - *Nature* **211**: 296 (1966)
185. Thomas P. J. and Dutton H. J. - *Anal. Chem.* **41**: 657 (1969)
186. Kirschner M. A. and Lipsett M. B. - *J. Lipid Res.* **6**: 7 (1965)
187. Blanc C., Huynh C.-T. and Espagno L. - *J. Chrom.* **28**: 177 (1967)
188. Blanc C., Huynh C.-T. and Espagno L. - *J. Chrom.* **28**: 194 (1967)
189. Fetzer J. C., Bloxham P. A. and Rogers L. B. - *Sep. Sci. Tech.* **15**: 49 (1980)
190. Gunter B. D. and Gleason J. D. - *J. Chrom. Sci.* **9**: 191 (1971)
191. Shepard A. T., Danielson N. D., Pauls R. E., Mahle N. H., Taylor P. J. and Rogers L. B. - *Sep. Sci.* **11**: 279 (1976)
192. Berger G., Prenant C., Sastre J. and Comar D. - *J. Label. Compd. Radiopharm.* **19**: 1486 (1982)
193. Colombo U., Gazzarrini F., Sironi G., Gonnantini R. and Tongiorgi E. - *Nature* **205**: 1303 (1965)
194. Bayer E., Nicholson G. and Sievers R. E. - *J. Chrom. Sci.* **8**: 467 (1970)
195. Meier-Augenstein W., Watt P. W. and Langhans C.-D. - *J. Chrom. A* **752**: 233 (1996)
196. Barrie A., Bricout J. and Koziel J. - *Biomed. Mass. Spectrom.* **11**: 583 (1984)
197. Berger G., Prenant C., Sastre J. and Comar D. - *Int. J. Appl. Radiat. Isot.* **34**: 1525 (1983)
198. Berger G., Prenant C., Sastre J. and Comar D. - *J. Label. Compd. Radiopharm.* **21**: 1294 (1984)
199. Dunstan R. H. - *Biomed. Env. Mass Spectrom.* **15**: 473 (1988)
200. Jeffrey A. M., Blobstein S. H., Weinstein I. B. and Harvey R. G. - *Anal. Biochem.* **73**: 378 (1976)
201. Jeffrey A. M. and Fu P. P. - *Anal. Biochem.* **77**: 298 (1977)
202. Weinstein I. B., Jeffrey A. M., Jennette K. W., Blobstein S. H., Harvey R. G., Harris C., Autrup H., Kasai H. and Nakanishi K. - *Science* **193**: 592 (1976)

203. Powell W. S. - *Anal. Biochem.* **115**: 267 (1981)
204. Powell W. S. - *Anal. Biochem.* **128**: 93 (1983)
205. Powell W. S. - *Adv. Prost. Thromb. Leuk. Res.* **11**: 207 (1983)
206. Turk J., Henderson W. R., Klebanoff S. J. and Hubbard W. C. - *Biochim. Biophys. Acta* **751**: 189 (1983)
207. Zijlstra F. J. and Vincent J. E. - *J. Chrom.* **311**: 39 (1984)
208. Pratt J. J. - *Ann. Clin. Biochem.* **23**: 251 (1986)
209. Hawkes J. S., James M. J. and Cleland L. G. - *Prostaglandins* **42**: 355 (1991)
210. Do U. H., Lo S.-L., Iles J., Rosenberger T., Tam P., Hong Y. and Ahern D. - *Prost. Leuk. and Essent. Fatty Acids* **50**: 335 (1994)
211. Unadkat J. D. and Rowland M. - *J. Chrom.* **261**: 298 (1983)
212. Napoli J. L., Mellon W. S., Fivizzani M. A., Schnoes H. K. and DeLuca H. F. - *Biochem.* **19**: 2515 (1980)
213. Perlman K., Schnoes H. K., Tanaka Y., DeLuca H. F., Kobayashi Y. and Taguchi T. - *Biochem.* **23**: 5041 (1984)
214. Halloran B. P., Bikle D. D. and Whitney J. O. - *J. Chrom.* **303**: 229 (1984)
215. Bikle D. D., Halloran B. P., Whitney J. O. and Hollis B. W. - *Biochem.* **23**: 6920 (1984)
216. Worth G. K. and Retallack R. W. - *Anal. Biochem.* **174**: 137 (1988)
217. Frenkel K., Goldstein M. S. and Teebor G. W. - *Biochem.* **20**: 7566 (1981)
218. Bodycote J. and Wolff S. - *Proc. Natl. Acad. Sci.* **83**: 4749 (1986)
219. Luo Y., Henle E. S., Chatopadhyaya R., Jin R. and Linn S. - *Methods in Enzymol.* **234**: 51 (1994)
220. Kudelin B. K., Gavrilina L. V. and Kaminski Y. L. - *J. Label. Compd. Radiopharm.* **31**: 723 (1992)
221. Van Der Krogt J. A., Van Valkenburg C. E. M. and Belfroid R. D. M. - *J. Chrom.* **427**: 9 (1988)
222. Kaspersen F. M., Funke G. W., Sperling E. M. G., van Rooy F. A. M. and Wagenaar G. N. - *J. Chem. Soc. Perkin Trans. II* 585 (1986)
223. Kudelin B. K., Gavrilina L. V. and Kaminski Y. L. - *J. Chrom.* **636**: 243 (1993)
224. Gerding T. K., Drenth B. F. H. and de Zeeuw R. A. - *Anal. Biochem.* **171**: 382 (1988)
225. Jones A. N., Simpson R. E. and Jenkins H. J. - *J. Label. Compd. Radiopharm.* **31**: 297 (1992)
226. Filer C. N., Fazio R. and Ahern D. G. - *J. Org. Chem.* **46**: 3344 (1981)
227. Yeung P. K. F., Hubbard J. W., Baker B. W., Looker M. R. and Midha K. K. - *J. Chrom.* **303**: 412 (1984)
228. Heys J. R. - Abstracts, Second Intern. Symp. Synth. Applic. Isot. Label. Compd., Kansas City, Mo., #PB-1 (1985)
229. Tanaka N. and Thornton E. R. - *J. Amer. Chem. Soc.* **98**: 1617 (1976)
230. Tanaka N. and Thornton E. R. - *J. Amer. Chem. Soc.* **99**: 7300 (1977)

231. Colson C. E. and Lowenstein J. M. - *Methods in Enzymol.* **72**: 53 (1981)
232. Adlof R. O. and Emken E. A. - *J. Chrom. A* **685**: 178 (1994)
233. Miles J. M. and Jensen M. D. - *J. Parenter. Enteral Nutr.* **15**: 90(s) (1991)
234. Van der Wal S.J. - *Chromatographia* **22**: 81 (1986)
235. Cantoni G. P. and Ferretti I. - *J. Chrom.* **122**: 287 (1976)
236. Kucera P. and Manius G. - *J. Chrom.* **216**: 9 (1981)
237. Kucera P. and Manius G. - *J. Chrom.* **219**: 1 (1981)
238. Jinno K., Hirata Y. and Hiyoshi Y. - *J. High Res. Chrom. Chrom. Commun.* **5**: 102 (1982)
239. Jinno K. - *J. High Res. Chrom. Chrom. Commun.* **5**: 364 (1982)
240. Van der Wal S.J. - *J. Liq. Chrom.* **8**: 2003 (1985)
241. Tayar N. E., Van de Waterbeemd H., Gryllaki M., Testa B. and Trager W. F. - *Int. J. Pharm.* **19**: 271 (1984)
242. Poulson S. R., Drever J. I. and Colberg P. J. S. - *Chemosphere* **35**: 2215 (1997)
243. Baweja R. - *Anal. Chim. Acta* **192**: 345 (1987)
244. Shi B. and Davis B. H. - *J. Chrom. A* **731**: 351 (1996)
245. Lockley W. J. S. - *J. Chrom.* **483**: 413 (1989)
246. Goromaru T., Sera S. and Ikeda H. - *Radioisotopes* **44**: 693 (1995)
247. Yu P. H., Bailey B. A., Durden D. A. and Boulton A. A. - *Biochem. Pharmacol.* **35**: 1027 (1986)
248. Yu P. H. and Davis B. A. - *Int. J. Biochem.* **20**: 1197 (1988)
249. Yu P. H. and Tipton K. F. - *Biochem. Pharmacol.* **38**: 4245 (1989)
250. Sniegowski L. T., White E. and Konash P. L. - *J. Label. Compd. Radiopharm.* **19**: 1081 (1982)
251. Kline W. F., Wise S. A. and May W. E. - *J. Liq. Chrom.* **8**: 223 (1985)
252. Masters C. F., Markey S. P., Mefford I. N. and Duncan M. W. - *Anal. Chem.* **60**: 2131 (1988)
253. Hsieh S. and Jorgenson J. W. - *Anal. Chem.* **69**: 3907 (1997)
254. Brown B. H., Neill S. J. and Horgan R. - *Planta* **167**: 421 (1986)
255. Wodzicki T. J., Abe H., Wodzicki A. B., Pharis R. P. and Cohen J. D. - *Plant Physiol.* **84**: 135 (1987)
256. Shibasaki H., Furuta T. and Kasuya Y. - *J. Chrom. B* **692**: 7 (1997)
257. Turk J., Stump W. T., Wolf B. A., Eason R. A. and McDaniel M. L. - *Anal. Biochem.* **174**: 580 (1988)
258. Baweja R. - *J. Liq. Chrom.* **9**: 2609 (1986)
259. Dueker S. R., Jones A. D., Smith G. M. and Clifford A. J. - *Anal. Chem.* **66**: 4177 (1994)
260. Bechalany A., Tayar N. E., Carrupt P. A., Testa B., Falconnet J. B., Cherrah Y., Bencheikroun Y. and Brazier J. L. - *Helv. Chim. Acta* **72**: 472 (1989)
261. Gerardy B. M., Poupaert J. H., Vandervorst D., Dumont P., Declercq J. P., Van Meerssche M. and Portoghese P. S. - *J. Label. Compd. Radiopharm.* **22**: 5 (1985)

262. Heys J. R. - *J. Chrom.* **407**: 37 (1987)
263. Heys J. R. - *Trans. Amer. Nuc. Soc.* **55**: 34 (1987)
264. Borel A. G. and Abbott P. S. - *J. Label. Compd. Radiopharm.* **28**: 771 (1990)
265. Taylor I. W., Ioannides C., Sacra P., Turner J. C. and Parke D. V. - *Biochem. Pharmacol.* **32**: 641 (1983)
266. Favier J. S., Wallkart J. and Kaspersen F. M. - *J. Label. Compd. Radiopharm.* **19**: 1125 (1982)
267. Kaspersen F. M., Van Acquoy J., Van de Laar G. L. M., Wagenaars G. N. and Funke C. W. - *Recl. Trav. Chim. Pays-Bas* **103**: 32 (1984)
268. De Ridder J. J. and Van Hal H. J. M. - *J. Chrom.* **121**: 96 (1976)
269. Szabo G. K., Pyllä R. J., Perchalski R. J. and Browne T. R. - *J. Chrom.* **535**: 271 (1990)
270. Farmer P. B., Foster A. B., Jarman M., Oddy M. R. and Reed D. J. - *J. Med. Chem.* **21**: 514 (1978)
271. Honma S., Iwamura S., Kobayashi R., Kawabe Y. and Shibata K. - *Drug Metab. Dispos.* **15**: 551 (1987)
272. Pearce D. W., Koshioka M. and Pharis R. P. - *J. Chrom. A* **658**: 91 (1994)
273. Baweja R. - *J. Chrom.* **369**: 125 (1986)
274. Baweja R. - *L. C. - G. C.* **4**: 1202 (1986)
275. Baweja R., Katz J. J. and Blake M. I. - *Trans. Amer. Nuc. Soc.* **55**: 35 (1987)
276. Dluznieski P. R. and Jorgenson J. W. - *J. High Res. Chrom. Chrom. Commun.* **11**: 332 (1988)
277. Bergqvist M. H. J. - *J. Chrom. A* **736**: 83 (1996)
278. Bezaud J. A. and Ouedraogo M. A. - *J. Chrom.* **196**: 279 (1980)
279. Van Eijk H. M. H., Rooyakkers D. R., Wagenmakers A. J. M., Soeters P. B. and Deutz N. E. P. - *J. Chrom. B* **691**: 287 (1997)
280. Matjeka E. R., Banks R. C., Mercer G. D., Musser B. and Vestal R. E. - *J. Label. Compd. Radiopharm.* **23**: 969 (1986)
281. Baumann P. Q., Ebenstein D. B., O'Rourke B. D. and Nair K. S. - *J. Chrom.* **573**: 11 (1992)
282. Wheeler W. J. and Blanchard W. B. - *J. Label. Compd. Radiopharm.* **31**: 495 (1992)
283. Braunsdorf R., Hener U., Przibilla G., Piecha S. and Mosandl A. - *Z. Lebensm. Unters. Forsch.* **197**: 24 (1993)
284. Gensler M. and Schmidt H.-L. - *Anal. Chim. Acta* **299**: 231 (1994)
285. Calini R. J. and Brenna J. T. - *J. Chrom. A* **757**: 307 (1997)
286. Tanaka N., Yamaguchi A., Araki M. and Kimata K. - *J. Amer. Chem. Soc.* **107**: 7781 (1985)
287. Tanaka N., Yamaguchi A., Hashizume K., Araki M., Wada A. and Kimata K. - *J. High Res. Chrom. Chrom. Commun.* **2**: 683 (1986)
288. Tanaka N., Hosoya K., Nomura K., Yoshimura T., Ohki T., Yamaoka R., Kimata K. and Araki M. - *Nature* **341**: 727 (1989)

289. Bidigare R. R., Kennicutt M. C., Keeney-Kennicutt W. L. and Macko S. A. - *Anal. Chem.* **63**: 130 (1991)
290. Araki M. and Tanaka N. - Japanese Pat. JP 61,293,535
291. Tanaka N. and Araki M. - *J. Amer. Chem. Soc.* **107**: 7780 (1985)
292. Tanaka N., Araki M. and Kimata K. - *J. Chrom.* **352**: 307 (1986)
293. Kimata K., Kobayashi M., Hosoya K., Araki T. and Tanaka N. - *J. Amer. Chem. Soc.* **118**: 759 (1996)
294. Tanaka N., Kimata K., Kobayashi M., Hosoya K. and Araki T. - *Kuromatogurafi* **17**: 150 (1996)
295. Kimata K., Hosoya K., Araki T. and Tanaka N. - *Anal. Chem.* **69**: 2610 (1997)
296. Makino T., Orlanopoulos M., You T.-P., Wu B., Mosher C. W. and Mosher H. S. - *J. Org. Chem.* **50**: 5357 (1985)
297. Pirkle W. H. and Gan K. Z. - *Tet. Asymm.* **8**: 811 (1997)
298. Mislow K., Graeve R., Gordon A. J. and Wahl G. H. - *J. Amer. Chem. Soc.* **86**: 1733 (1964)
299. Cveticanovic R. J., Duncan F. J., Falconer W. E. and Irwin R. S. - *J. Amer. Chem. Soc.* **87**: 1827 (1965)
300. Bigeleisen J. - *J. Chem. Phys.* **34**: 1485 (1961)
301. Bigeleisen J., Ribnikar S. V. and Van Hook W. A. - *J. Chem. Phys.* **38**: 489 (1963)
302. Bigeleisen J., Stern M. J. and Van Hook W. A. - *J. Chem. Phys.* **38**: 497 (1963)
303. Stern M. J., Van Hook W. A. and Wolfsberg M. - *J. Chem. Phys.* **39**: 3179 (1963)
304. Davis R. T. and Schiessler R. W. - *J. Phys. Chem.* **57**: 966 (1953)
305. Van der Linde W. and Robertson R. E. - *J. Amer. Chem. Soc.* **86**: 4505 (1964)
306. Northeott D. and Robertson R. E. - *J. Phys. Chem.* **73**: 1559 (1969)
307. Meier-Augenstein W. - *L.C.-G.C.* **15**: 244 (1997)

Curve Fitting for Restoration of Accuracy for Overlapping Peaks in Gas Chromatography/Combustion Isotope Ratio Mass Spectrometry

Keith J. Goodman and J. Thomas Brenna*

Division of Nutritional Sciences, Cornell University, Ithaca, New York 14853

The effect of graded degrees of overlap on high-precision and -accuracy carbon isotope ratios determined by gas chromatography/combustion isotope ratio mass spectrometry (GCC/IRMS) is reported. Overlapping peaks of closely matched isotope ratio (difference $\delta^{13}\text{C}_{\text{PDB}} < 1\%$) were analyzed by the conventional vertical drop summation algorithm and by curve fitting using the Levenberg-Marquardt algorithm. The conventional algorithm resulted in systematic bias related to degree of overlap even though precision was not noticeably affected. The exponentially modified Gaussian (EMG) and Haarhoff-VanderLinde (HVL) functions were found to model GCC/IRMS peaks satisfactorily. Useful models over a wide range of overlap were obtained by applying consecutive HVL/HVL or HVL/EMG functions to overlapping peaks. Accuracy was improved in most cases and was never degraded. This study demonstrates the presence of subtle bias in isotope ratio determinations of overlapping peaks and the ability of automated curve fitting to compensate for these biases.

High-precision compound-specific isotope analysis (CSIA) is rapidly becoming an important tool in a variety of fields as diverse as organic geochemistry and biomedicine. Gas chromatography/combustion isotope ratio mass spectrometry (GCC/IRMS) and the recent introduction of liquid-based CSIA¹ facilitate online separation, combustion, and analysis of carbon isotopes in organic molecules. CSIA has been used primarily to investigate small changes in carbon isotope ratios that occur due to natural processes,^{2,3} although recently the technique has been used for enriched species as well.^{4,5}

In GCC/IRMS, CO_2 generated by combustion of GC effluent is analyzed by a multicollector mass spectrometer. CO_2 is continuously monitored at masses 44 ($^{12}\text{C}^{16}\text{O}_2$), 45 ($^{13}\text{C}^{16}\text{O}_2 + ^{12}\text{C}^{17}\text{O}^{16}\text{O}$), and 46 ($^{12}\text{C}^{18}\text{O}^{16}\text{O}$). A peak in each detector trace appears for each eluting compound, and thus each trace resembles the output of a flame ionization detector (FID). At the conclusion of the run, software must identify peak start/stop, subtract background levels, calculate peak areas, and calculate area ratios according to theoretical considerations.⁶ Highly precise and accurate isotope ratio determinations depend on consistent peak and background definitions for each of the three detection channels. Total

error (precision and accuracy) for isotope ratios determined in this way is routinely less than 0.05% relative standard deviation (RSD) for well-resolved peaks.

Analysis of complex mixtures often results in overlapping chromatographic peaks. Specific requirements for auxiliary preparation devices (e.g., furnaces, dryers) for GCC/IRMS results in a large number of connectors between the chromatography column and the detector, which influence peak shape and generally reduce chromatographic efficiency.^{7,8} Resolution is also limited by the restriction of He as carrier gas, compared with H_2 . As a result, mixtures that are fully resolved by conventional GC-FID are not resolved by GCC/IRMS. For these reasons, chromatography in GCC/IRMS tends to be more subject to overlaps than conventional GC.

Mathematical curve fitting for quantitative analysis of overlapping peaks has been reported extensively in the chromatography literature. Curve fitting for well-resolved peaks is thought to offer several advantages over summation methods, including straightforward definition of peak limits and less susceptibility to noisy baselines.⁹⁻¹¹ Previous studies focused on single detector systems and have the explicit purpose of providing improved quantitative results compared with nonfitting techniques. To date, no systematic studies of the effect of overlap on high-precision isotope determinations by GCC/IRMS have appeared.

Traditional chromatograms may be thought of as a collection of well-resolved singlets with a few doublets of varying degrees of overlap. The recovery of information from doublets is the subject of this work. It is assumed that triplets and higher multiplets, although rare according to theoretical considerations,¹² will be resolved chromatographically.

Several characteristics of a curve-fitting algorithm are desirable. (1) The algorithm should be "rugged". That is, it should yield acceptable results over a wide range of operating conditions, preferably in an automated system. (2) It must run rapidly with reasonable hardware requirements. (3) It should be useful over a wide range of overlap encountered for real samples. (4) Finally, it should improve analytical figures of merit for most peaks; those that it does not improve it

- (1) Calmi, R. J.; Brenna, J. T. *Anal. Chem.* 1993, 65, 3497-3500.
- (2) Matthews, D. E.; Hayes, J. M. *Anal. Chem.* 1978, 50, 1465-1473.
- (3) Rautenschlein, M.; Habfast, K.; Brand, W. *Stable Isotopes in Paediatric Nutritional and Metabolic Research*; Intercept Limited: Andover, UK, 1990.
- (4) Goodman, K. J.; Brenna, J. T. *Anal. Chem.* 1992, 64, 1088-1095.
- (5) Guo, Z. K.; Luke, A. H.; Lee, W. P.; Schoeller, D. *Anal. Chem.* 1993, 65, 1954-1959.
- (6) Santrock, J.; Studley, S. A.; Hayes, J. M. *Anal. Chem.* 1985, 57, 1444-1448.

- (7) Guiochon, G. *J. Gas Chromatogr.* 1964, 139-145.
- (8) Maynard, V.; Grushka, E. *Anal. Chem.* 1972, 44, 1427-1434.
- (9) Anderson, A. H.; Gibb, T. C.; Littlewood, A. B. *Anal. Chem.* 1970, 42, 434-440.
- (10) Anderson, A. H.; Gibb, T. C.; Littlewood, A. B. *J. Chromatogr. Sci.* 1970, 8, 640-646.
- (11) Dyson, N. *Chromatographic Integration Methods*; The Royal Society of Chemistry: Cambridge, UK, 1990.
- (12) Davis, J. M.; Giddings, J. C. *Anal. Chem.* 1983, 55, 418-424.

should not degrade. We report here an experimental study of the effects of overlapping peaks on accuracy and precision of GCC/IRMS-determined isotope ratios and a curve-fitting strategy applicable to recovery of isotope information from overlapping peaks.

EXPERIMENTAL SECTION

Instrumentation. A Finnigan MAT 252 high-precision GIRMS instrument interfaced to a Varian 3400 GC via a ceramic combustion furnace was used for the analysis. A detailed description of this instrumentation can be found elsewhere.⁴ Briefly, the effluent from the capillary GC column enters a furnace and is combusted quantitatively to CO₂. After drying, analyte-derived CO₂ is admitted into the electron impact ion source of the high-precision GIRMS system. The mass spectrometer is operated at its full accelerating potential of 10 kV and a source chamber pressure of 4×10^{-6} Torr. Faraday cup detectors with dedicated amplifiers and counters continuously monitor each of the three major ion beams. For this work, the integration time was 0.25 s, as is routine in our laboratory. The GC was operated with the injector ($T = 250$ °C) in split mode with a split ratio of 20:1 and He carrier flow of 45 cm/s through the column. A J&W DB-WAX 30 m \times 0.32 mm \times 0.5 μ m fused silica capillary column was used. The oven temperature program was isothermal.

Test Chromatograms. Methyl tridecanoate (Me13:0) and butylated hydroxytoluene (BHT; Sigma Chemical Co, St. Louis, MO) were used as test compounds. Stock solutions of each pure component were prepared in bulk and used throughout the experiment to ensure the isotopic integrity of the samples. Mixtures were prepared from the stock solutions in the mass ratios 1:1, 10:1, and 1:10. These ratios were chosen as representative of the extremes of mass ratios to which this work applies. Also, for integration routines that employ both perpendicular drop and tangent-skim algorithms, an absolute abundance ratio of 10 commonly represents the decision threshold between the two methods.¹³ Concentrations were adjusted to 5 μ g/ μ L for each component of the 1:1 mixture and the more abundant components of the unequal mixtures (1:10 and 10:1). The concentrations of the less abundant components of the unequal mixtures were adjusted to 0.5 μ g/ μ L. An injection volume of 1 μ L yielded a peak signal of 3.5 V in the mass 44 channel for the 1:1 components and the major components of the unequal mixtures.

Graded degrees of overlap may be produced by double injection or by manipulation of chromatographic parameters. The chief advantage of double injection, used primarily for liquid chromatography (LC), is optimal preservation of peak shape.¹⁴ This approach is not practical for GC since peaks are sharp and maintaining reproducibility of overlap is difficult. For this reason, graded overlap was produced by increasing the column temperature from 220 to 250 °C for isothermal runs. This method generated highly reproducible overlap for the eluting compounds by degrading resolution. Peak shapes were not noticeably different for individual compounds run at each temperature, although least-squares fits did show some small distortion, as reflected by r^2 . This distortion was well within that observed for injection of widely differing quantities

under identical conditions. The degree of overlap is expressed as "%valley", calculated as $100(\text{valley height})/(\text{peak height})$.¹⁴ Conditions were established to produce 10, 40, and 70% valley for the 1:1 mixture. A 70% valley is observed in dense chromatograms of complex mixtures and should be particularly useful for GCC/IRMS applications to enriched tracer molecules requiring lower precision. The 10:1 and 1:10 mixtures were analyzed under each set of conditions used for the 1:1 case, and in subsequent discussion are designated 10, 40, or 70% valley with respect to the 1:1 case. Pure compounds were run separately under conditions used for 10% valley and plotted as "0% valley". Me13:0 eluted first under these conditions.

Data Analysis: Conventional Method. Data was analyzed by the conventional method using vendor-provided software (Finnigan ISODAT). The peak detect algorithm processes only one data channel for the detection and definition of peak start and stop. Peaks are detected by calculating the slope of a rolling regression line drawn through five consecutive data points and comparing the result to a user-defined threshold slope ("slope sensitivity"). Peak stops are set when the slope falls below threshold, after a peak start has been detected. The slope sensitivity was set to a single intermediate value that produces satisfactory results in our hands for well-resolved peaks at signal-to-noise ratios above ~ 50 . Backgrounds are determined as the average of four adjacent points beginning five points before the start of the peak. Once the peak start and stop have been defined for a single channel, the values are extrapolated to the other two channels. Peak maxima are determined for all channels and a time shift is used to correct for chromatographic separation of isotopes.^{3,15} Peak areas are calculated by summing the difference between baseline and signal for the region defined by the peak start and stop. Ratios and associated calculations are made using areas produced for each peak.¹⁶ For overlapping peaks, the valley minimum is assigned as the leading peak stop and the trailing peak start. When applied to overlapping peaks, the routine resembles the "perpendicular drop" method.^{11,13} Summing of consecutive readings between peak limits after subtraction of baseline is referred to as the "summation" method.¹⁷

The ratios of areas corresponding to individual components are taken for each trace (44, 45, and 46) to produce R45 (area 45/area 44) and R46 (area 46/area 45). R45 and R46 are both required for calculating $\delta^{13}\text{C}$, as R46 is used for an ^{17}O correction that subtracts the contribution of [$^{12}\text{C}^{17}\text{O}^{16}\text{O}$] to the m/z 45 signal.⁶ For compounds of low oxygen content, such as those considered here, most of the O of CO₂ is derived from the combustion furnace and is therefore invariant. The small contribution from the test compounds, Me13:0 and BHT, is also of identical isotopic composition within experimental error. Calculations show that relatively large changes in the mass 46 channel do not appreciably alter the $\delta^{13}\text{C}$ calculations. For these reasons, and to simplify the calculations for the curve fitting, traces of mass 44 and 45 only were fitted and the data will be presented without correction for ^{17}O .

The standard notation for expression of high-precision gas isotope ratio mass spectrometry results is the $\delta^{13}\text{C}$ notation,

(13) Papas, A. N.; Tougas, T. P. *Anal. Chem.* 1990, 62, 234-239.

(14) Jeannotte, M. S.; Foley, J. P. *J. Chromatogr.* 1989, 461, 149-163.

(15) Freeman, K. H.; Hayes, J. M.; Trendel, J. M.; Albrecht, P. *Nature* 1990, 343, 254-256.

(16) Ricci, M., private communication, 1993.

(17) Anderson, D. J.; Walters, R. R. *J. Chromatogr. Sci.* 1984, 22, 353-359.

which requires the ^{17}O correction. In this work, we use $\delta 45$ defined as

$$\delta 45 = \left(\frac{R_{\text{SPL}} - R_{\text{PDB}}}{R_{\text{PDB}}} \right) \times 1000 \quad (1)$$

where R_{SPL} and R_{PDB} are the m/z 44 and 45 signal ratios corresponding to the sample and standard, Pee Dee Belemnite, respectively, with $R_{\text{PDB}} = 0.011\,237\,2 \pm 0.000\,009\,0$.¹⁸

Data Analysis: Curve Fitting. Raw data files were converted to ASCII files, containing four columns of data corresponding to time, and counts (arbitrary units) for each of three detectors m/z 44, 45, and 46. A BASIC program was then used to split the data files into three separate files, each containing time and one of the signals. Data fitting was performed using a Compaq 386/20e desktop computer equipped with a math coprocessor, 4 Mbytes of RAM, and a 210-Mbyte hard drive. The DOS computer program Peakfit 3.0 (Jandel Scientific), an implementation of the Levenberg-Marquardt algorithm, was used for all curve fitting.¹⁹ Given a set of initial values, this algorithm systematically searches parameter space to find parameters yielding the minimum error sum of squares. A single chromatogram was fitted manually using each of several test chromatographic functions to establish initial parameter values. These values were then used as initial values for all subsequent fits. Chromatograms for each detection channel were fitted in a batch-processing mode using several functions. The batch mode permits a user-selectable prefit routine that fits every other point as a rapid "coarse" fit, followed by fine adjustments using all the data. This procedure results in more rapid fitting than use of all the data from the outset. Each function is expressed using peak area as an independent, adjustable parameter. Isotope ratios were calculated from the best-fit peak area parameter.

A variety of functions have been proposed for modeling of chromatographic peaks; therefore it was necessary to determine which functions most satisfactorily modeled GCC/IRMS data. Functions were evaluated based on the fit parameters r^2 and mean square ratio (F statistic), and comparing the calculated mass and area ratios to the known ratios of the mixtures. Time for fitting was also taken into account.

Chromatographic elution profiles previously have been modeled as a Gaussian distribution. Under real chromatographic conditions, peaks are often skewed and not modeled well by symmetric functions such as a Gaussian.²⁰⁻²⁴ Peak models that take asymmetry into account improve accuracy and precision relative to symmetric models.²⁵⁻³² The exponentially modified Gaussian (EMG) function, a mathematical convolution of a Gaussian with an exponential decay, has

proven to be an acceptable model for real (asymmetric) chromatographic peaks, especially when extra column effects are present.^{10,33} The form of this function is

EMG

$$f(x) = \frac{a_0}{2a_3} \exp \left[\frac{a_2^2}{2a_3^2} + \frac{a_1 - x}{a_3} \right] \left[\operatorname{erf} \left(\frac{x - a_1}{2^{1/2}a_2} - \frac{a_2}{2^{1/2}a_3} \right) + 1 \right] \quad (2)$$

where a_0 is area, a_1 is elution time, a is width of the Gaussian, a_3 is the exponential constant which determines distortion in this model, and erf is the error function. Estimates of peak area, chromatographic efficiency, and maximum attainable efficiency, can be obtained from these function parameters.^{28,29,31} The EMG model, one of the most commonly employed chromatographic functions for modeling GC peaks, has also been successfully applied to situations where baseline resolution has not been achieved.³³⁻³⁵

Other functions considered here are the (a) Haarhoff-VanderLinde (HVL), which was developed for nonideal gas chromatography and is capable of modeling peaks that exhibit "fronting" or tailing,²⁵ (b) Giddings (GID), a function designed for conditions in which diffusion and extracolumn effects are absent and different kinetic rates of adsorption and desorption are the primary sources of band broadening,³⁶ and (c) nonlinear chromatographic (NLC), which was recently considered for modeling ideal δ function analyte loading.³⁷ The forms of these equations are as follows:

HVL

$$f(x) = \frac{a_0 a_2}{a_1 a_3 (2\pi)^{1/2}} \frac{\exp \left(-\frac{1}{2} \left(\frac{x - a_1}{a_2} \right)^2 \right)}{\left[\exp \left(\frac{a_1 a_3}{a_2^2} \right) - 1 \right] + \frac{1}{2} \left[1 + \operatorname{erf} \left(\frac{(x - a_1)/a_2}{2^{1/2}} \right) \right]} \quad (3)$$

GID

$$f(x) = \frac{a_0}{a_2} \frac{a_1^{1/2}}{x} I_1 \left(\frac{2(a_1 x)^{1/2}}{a_2} \right) \exp \left(\frac{-x - a_1}{a_2} \right) \quad (4)$$

NLC

$$f(x) = \frac{a_0}{a_2 a_3} \left[1 - \exp \left(-\frac{a_3}{a_2} \right) \right] \left[\frac{\frac{a_1^{1/2}}{x} I_1 \left(\frac{2(a_1 x)^{1/2}}{a_2} \right) \exp \left(\frac{-x - a_1}{a_2} \right)}{1 - \exp \left(-\frac{a_3}{a_2} \right)} \right] \quad (5a)$$

with

- (33) Wu, N. S.; Cai, C. P.; Yang, Y. *Chromatographia* 1990, 30, 220-222.
 (34) Binsheng, L.; Feichang, L. *HRC CC, J. High Resolut. Chromatogr. Chromatogr. Commun.* 1987, 10, 449-454.
 (35) Foley, J. P. *J. Chromatography* 1987, 384, 301-313.
 (36) Giddings, J. C. *Dynamics of Chromatography*; Marcel Dekker: New York, 1965.
 (37) Wade, J. L.; Bergold, A. F.; Carr, P. W. *Anal. Chem.* 1987, 59, 1286-1295.

- (18) Craig, H. *Geochem. Cosmochim. Acta* 1957, 22, 133-149.
 (19) Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes: The Art of Scientific Computing*; Cambridge University Press: New York, 1986.
 (20) Schmauch, L. *J. Anal. Chem.* 1959, 31, 225-230.
 (21) Johnson, H. W., Jr.; Stross, F. H. *Anal. Chem.* 1959, 31, 357-365.
 (22) Giddings, J. C. *Anal. Chem.* 1963, 35, 1999-2002.
 (23) Gladney, H. M.; Dowden, B. F.; Swalen, J. D. *Anal. Chem.* 1969, 41, 883-888.
 (24) Pauls, R. E.; Rogers, L. B. *Anal. Chem.* 1977, 49, 625-628.
 (25) Haarhoff, P. C.; Van Der Linde, H. J. *Anal. Chem.* 1966, 38, 573-582.
 (26) Chesler, S. N.; Cram, S. P. *Anal. Chem.* 1973, 45, 1354-1359.
 (27) Pauls, R. E.; Rogers, L. B. *Sep. Sci.* 1977, 12, 395-413.
 (28) Barber, W. E.; Carr, P. W. *Anal. Chem.* 1981, 53, 1939-1942.
 (29) Foley, J. P.; Dorsey, J. G. *Anal. Chem.* 1983, 55, 730-737.
 (30) Foley, J. P.; Dorsey, J. G. *J. Chromatogr. Sci.* 1984, 22, 40-46.
 (31) Foley, J. P. *Anal. Chem.* 1987, 59, 1984-1987.
 (32) Jeansonne, M. S.; Foley, J. P. *J. Chromatogr. Sci.* 1991, 29, 258-266.

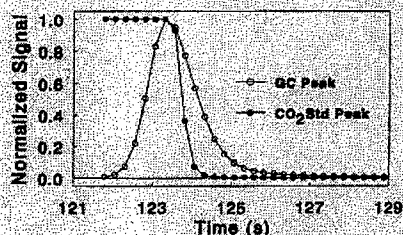


Figure 1. Signal from mass 44 channel illustrating signal decay of GC peak and CO₂ standard after closing of a valve. The GC signal decays ~3-fold slower than that resulting from the valve closing.

$$T(u, v) = e^{-u} \int_0^v e^{-t} I_0((2vt)^{1/2}) dt \quad (5b)$$

where a_0 is peak area, a_1 is elution time, a_2 is peak width, a_3 is distortion (for HVL and NLC), and I_n are modified Bessel functions of the first kind.

Peak Shapes. The degradation of chromatographic efficiency by dead volumes has been considered previously.^{7,8} Most chromatographic detectors are placed at the exit of the chromatography column to minimize dead volumes and unnecessary broadening due to diffusion after the separation. In GCC/IRMS instrumentation, additional connections are required to interface the capillary column with the mass spectrometer. In the present instrument, this corresponds to eight connections between the GC column and the mass spectrometer and includes two changes in tubing diameter to accommodate a furnace and water trap. An open split, a device used to moderate pressure, is placed in-line prior to the mass spectrometer and peak shape is sensitive to the flow at this split. Finally, the tight ion source of GIRMS instruments is designed to maximize residence time, in order to maximize sensitivity.

The net effect of this hardware is to distort peak shapes appreciably from that emerging from the GC column. The trailing section of a sharp, fast-eluting, well-behaved GC peak is plotted in Figure 1, along with the falloff in signal from closing of the valve that admits calibrated CO₂ directly to the ion source. The half-life for the calibrated CO₂ signal is $t_{1/2} = 170$ ms compared with $t_{1/2} = 530$ ms for the peak and demonstrates that the ion source responds more rapidly to changes in signal than required for most capillary GC peaks. The source of peak broadening resides in the chromatographic separation and interface, rather than the mass spectrometer ion source.

RESULTS AND DISCUSSION

The chromatograms for the various test mixtures and degrees of overlap are presented in Figure 2. The absolute retention times from chromatogram to chromatogram have been adjusted for comparison purposes but the peak shapes and widths with respect to time are unaltered.

Single Peaks. The four separate functions, EMG, GID, HVL, and NLC were screened for goodness of fit and fitting time. Both the r^2 and F statistics are commonly employed to evaluate goodness of fit. R^2 alone is not sufficient to define the quality of fit, as it is an index of noise as well as degrees of freedom in the model relative to the number of data

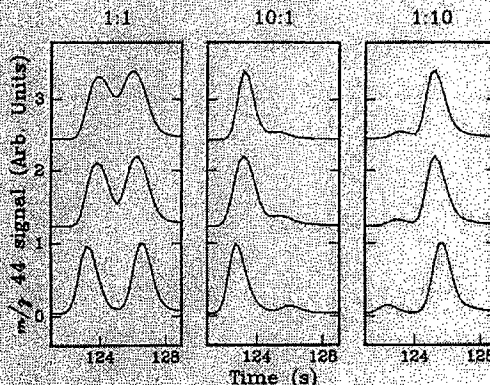


Figure 2. Chromatograms for the three mixtures and three overlap conditions investigated in this work. Me13:0 elutes prior to BHT in these chromatograms.

Table 1

| | r^2 | F |
|-----|---------------------|---------------|
| EMG | 0.999 48 ± 0.000 26 | 41864 ± 14615 |
| HVL | 0.997 85 ± 0.000 77 | 9627 ± 2779 |
| NLC | 0.997 86 ± 0.000 76 | 9762 ± 2837 |
| GID | 0.991 20 ± 0.003 74 | 4167 ± 3820 |

* Mean ± SD ($n = 6$).

points.^{38,39} The F statistic, however, is adversely affected by irrelevant degrees of freedom in the model.³⁸ Therefore, both the r^2 and F statistics were calculated to evaluate goodness of fit, and residuals were plotted for inspection.

Six typical chromatograms were fitted with each function to evaluate the appropriate function to model GCC/IRMS peaks. No less than 40 data points were used for each fit. The data are presented in Table 1 as means and standard deviations. The EMG fits resulted in significantly greater mean r^2 and F statistics (pairwise t , 95% confidence). The residual plots for the EMG fits were random. The HVL and NLC resulted in comparable fits, which were poorer than the EMG. Inspection of the residuals indicated that the peak tail was not modeled well for either the HVL or NLC. In addition, the NLC function required 66 s per mass channel compared with 25 s for the H function, a substantial 2.6-fold difference. The GID function gave the poorest fit based on both r^2 and F , as might have been anticipated based on the assumptions of that model and the present application. The isotope ratios calculated from fits with each function did not reveal any clear trend in absolute value or precision. Based on these results, the GID and NLC functions were eliminated from further consideration and the EMG and HVL functions were applied to unresolved peaks. For convenience, we refer to these functions by their first letters in the following.

Fitting Characteristics of Overlapping Peaks. The E and H functions were screened for goodness of fit for overlapping peaks. In addition to r^2 and F , the accuracy of the peak area ratios was used as an index of goodness of fit. At higher degrees of overlap the EE combination broke down. (XY refers to the application of function X to the first eluting peak

(38) Gans, P. *Data Fitting in the Chemical Sciences*; John Wiley & Sons: New York, 1992.

(39) Lancaster, P.; Salkauskas, K. *Curve and Surface Fitting*; Academic Press: New York, 1986.

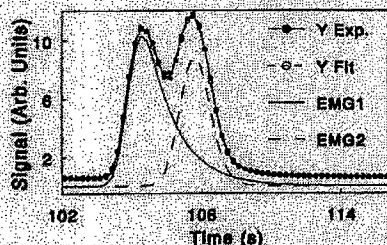


Figure 3. Best fit for the application of the EMG/EMG function combination to a 1:1, 70% valley chromatogram. The tail of the leading EMG function extends into the second peak, leading to erroneous abundance and isotope ratios.

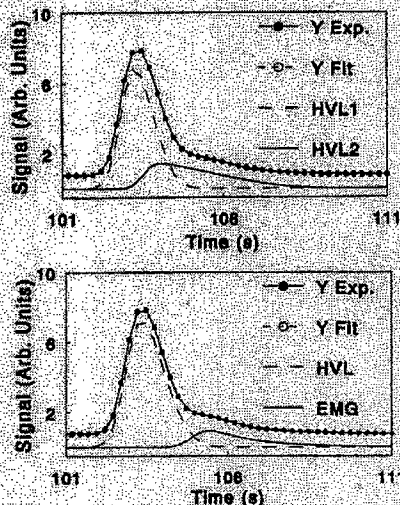


Figure 4. (a, top) Best fit for the HVL/HVL combination to a 10:1, 70% valley chromatogram. The leading edge of the trailing HVL function extends into the first peak. (b, bottom) Best fit for the HVL/EMG combination to the same data.

of a doublet, with Y applied to the trailing peak.) The EE fit of the 1:1 70% valley is presented in Figure 3. This fit resulted in a superior r^2 and F statistic compared with the HH combination. The first E function, however, distorts and tails excessively into the second peak, resulting in inaccurate peak areas. In contrast, the HH combination yielded a poorer r^2 but better estimates for the peak areas. Application of the HH combination to the other overlap chromatograms revealed that the HH combination did not provide accurate estimates for the 10:1 70% valley, shown in Figure 4a. The area for the second H peak area was overestimated because it fronted into the first peak to compensate for inability of the H function to model the tail of the first peak. Since the E was susceptible to errors due to excessive tailing and the H due to fronting, the HE combination was tested. A plot of the HE combination model for a 10:1 70% valley is presented in Figure 4b. In this plot, all experimental points are coincident with those of the model. This combination did not show any obvious fronting or tailing distortions for any of the conditions and, therefore, was used along with HH and EE for fitting all overlaps. The fourth and remaining combination of EH was evaluated for completeness.

The nine overlap conditions of Figure 2 were fitted by use of each of these four combinations of functions. Three or four replicate chromatograms were obtained and fitted for

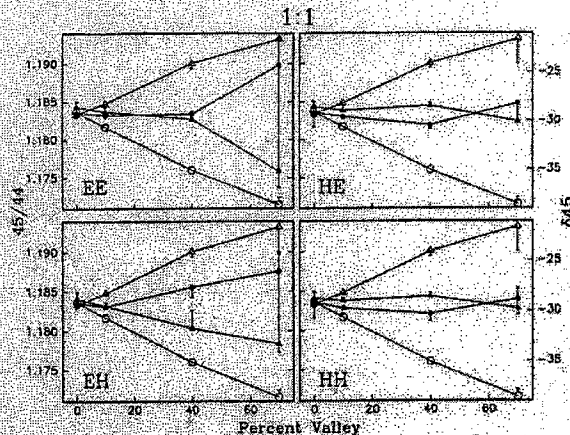


Figure 5. Isotope ratios obtained by summation and fitting for equal abundance mixtures. Open symbols refer to summation data and are repeated in all cells for comparison. Closed symbols refer to fitted data. Circles refer to Me13:0, which elutes earlier than BHT, represented by squares. Error bars are 95% confidence limits (using t statistic).

each overlap condition. Fits were evaluated for quality of (a) isotope ratios and (b) single-channel (m/z 44) area ratios, for quantitative analysis. To evaluate accuracy, results were compared to those obtained by summation and offline combustion. All differences in means were tested for significance by use of the pairwise t test at a 95% confidence level.⁴⁰

Isotope Ratios: Equal Abundance. Figure 5 presents R_{45} and corresponding δ_{45} obtained by summation and fitting for the four combinations of functions. Each data point represents a mean of at least three replicates fitted independently. The summation data are repeated in each cell for convenient comparison. Error bars represent 95% confidence limits, and when absent, they are contained within the symbol. The δ_{45} s and standard deviations for these data are presented in Table 2.

Isotope ratios for all degrees of overlap are significantly different when determined by summation than for 0% valley and appear to be systematically related to degree of overlap. At 70% valley, the apparent isotope ratio of the leading peak (Me13:0) is depleted by about $\delta_{45} = \sim 10\%$, while the isotope ratio of the trailing peak (BHT) is enriched by a corresponding level. This unexpected result is observed even though the difference in carbon isotope ratio between these compounds is only $\delta_{45} = -0.30$. It is significant to note that the precision for most of these highly inaccurate ratios is not seriously degraded, suggesting that precision is not a good indicator for accuracy for the summation method.

Fitted and summation data for the leading peak at 0% valley are not significantly different for any of the functions. Significant differences were detected for 0% valley trailing peak using the EE and EH fits ($\delta_{45} = -26.67 \pm 0.189$) compared with summation data ($\delta_{45} = -27.51 \pm 0.097$).

For all function combinations, the fitted ratios are of significantly improved accuracy relative to the summation ratios for 10 and 40% valley. All functions except the EE combination resulted in improved accuracy at the 70% level.

(40) Snedecor, G. W.; Cochran, W. G. *Statistical Methods*, 8th ed.; Iowa State University Press: Ames, IA, 1989.

| Table 2 | | |
|----------|---------------------|------------------|
| % valley | Me13:0 ^a | BHT ^a |
| SUM | | |
| 0 | -27.21 ± 0.46 | -27.51 ± 0.097 |
| 10 | -29.48 ± 0.17 | -26.98 ± 0.26 |
| 40 | -34.53 ± 0.13 | -23.00 ± 0.54 |
| 70 | -37.99 ± 0.33 | -20.12 ± 1.1 |
| EE | | |
| 0 | -27.28 ± 0.18 | -26.67 ± 0.19 |
| 10 | -27.84 ± 0.16 | -27.49 ± 0.34 |
| 40 | -27.72 ± 0.26 | -28.31 ± 0.49 |
| 70 | -22.24 ± 5.5 | -34.16 ± 6.1 |
| HE | | |
| 0 | -27.26 ± 0.70 | -26.67 ± 0.19 |
| 10 | -28.03 ± 0.16 | -27.36 ± 0.38 |
| 40 | -28.99 ± 0.29 | -28.80 ± 0.21 |
| 70 | -26.56 ± 0.56 | -28.59 ± 0.76 |
| HH | | |
| 0 | -27.26 ± 0.70 | -26.83 ± 0.12 |
| 10 | -28.17 ± 0.13 | -27.39 ± 0.19 |
| 40 | -28.83 ± 0.48 | -26.93 ± 0.24 |
| 70 | -27.27 ± 0.69 | -28.26 ± 0.88 |
| EH | | |
| 0 | -27.28 ± 0.18 | -28.83 ± 0.12 |
| 10 | -27.95 ± 0.14 | -27.91 ± 0.24 |
| 40 | -25.87 ± 0.70 | -30.34 ± 1.2 |
| 70 | -24.14 ± 3.56 | -32.07 ± 4.0 |

^a $\delta 45 \pm$ SD.

The EE combination gives very poor precision at 70% valley. The E function is known to break down at high degrees of overlap (>45% valley), and so this result is consistent with previous reports. This appears to be due to the tendency of this function to tail excessively from the leading peak into the trailing peak. The EH combination is consistent with this interpretation as it results in the poorest precision and accuracy at the 40% valley. Isotope ratios determined using the EE and EH functions cross over between 10 and 40% valley. In contrast, the HE and HH functions recover isotope ratios similarly to one another and more accurately than the other combinations. The isotope ratios for the HE and HH cross over between 40 and 70% valley. For this data set these functions appear to provide superior recovery of isotope ratios.

We have previously considered the accuracy of GCC/IRMS carbon isotope determinations referenced to conventional combustion and dual-inlet GIRMS analysis.⁴ An offset of up to $\delta^{13}\text{C} = 1\%$ is observed for GCC/IRMS analyses referenced to calibrated CO_2 . For this reason, the use of isotopically calibrated standards within the chromatography mixture has been suggested.⁴ In any case, the offset observed for fitted data up to 40% valley is small and within this accuracy limit.

Isotope Ratios: Unequal Abundance. Fit and summation results for the 10:1 mixture is shown in Figure 6a. The scaling is identical to that used in Figure 5 for comparison, and again the summation results are plotted for all fitting combinations. The summation peak detection algorithm detected two peaks for the 40% valley in only a single case and failed to detect two peaks for any of the 70% valley replicates. However, the presence of the smaller peak significantly lowered the calculated isotope ratio for the large peak in the 10% case. In the single 40% valley case the presence of the smaller peak altered the major peak by $\delta 45 = \sim 2\%$. The summation results for the smaller peak result in substantially greater degradation of both precision and accuracy than for the larger peak.

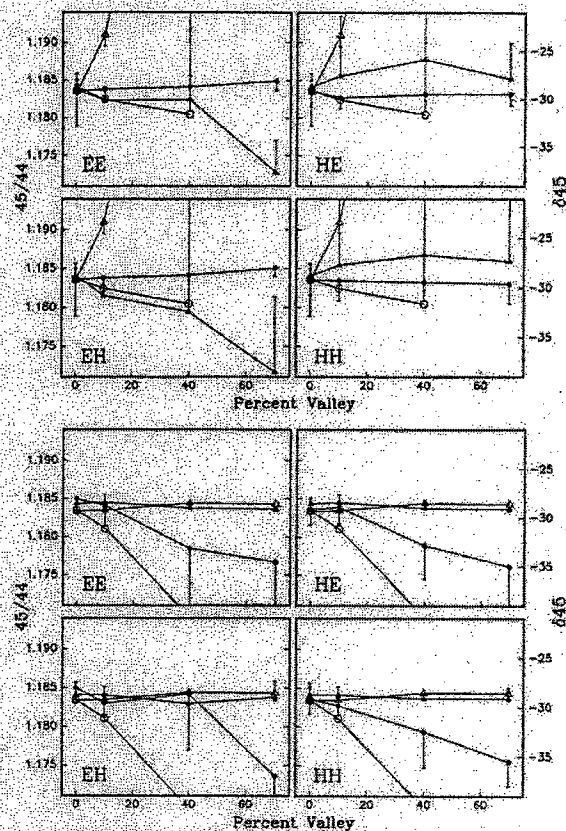


Figure 6. Isotope ratios obtained by summation and fitting for the (a, top) 10:1 and (b, bottom) 1:10 abundance mixture. The symbol key is given in the legend to Figure 5.

No significant differences are detected between fitted and summation ratios for either 0 or 10% valley for the leading peak, while the EE and EH combinations provide similar ratios, which significantly improve the accuracy of the smaller trailing peak. The HE and HH combinations give similar results for which isotope ratios are reversed relative to the other combinations. Not for any fitted case is the accuracy degraded relative to summation.

Data for the 1:10 mixture is presented in Figure 6b. In this case, the summation peak detect algorithm successfully identified all peaks. Summation and fitted ratios for the larger trailing peak are significantly different at the 0% valley only. In contrast to the 10:1 case, the precision and accuracy of the isotope ratio of the larger peak is affected by less than 1% for all degrees of overlap using the summation method.

For the smaller leading peak, summation ratios for all degrees of overlap are significantly different from the 0% valley, and the trend toward lower isotope ratios is significant, though smaller than the trend of the smaller peak in the 10:1 data. At the 10 and 40% valleys, the improvement in ratio for the fitted data is statistically significant.

Quantitative Analysis. Ratios of fitted peak areas for the mass 44 channel were constructed to evaluate the preservation of quantitative information. Area ratios were constructed for summation and fitted data using the leading peak in the numerator and are presented graphically in Figure 7 for the equal abundance data (1:1). The summation ratios show a

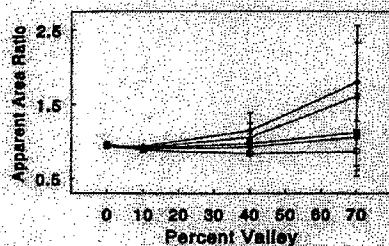


Figure 7. Area ratios for the equal abundance data calculated using the mass 44 signal: Me13:0/BHT, 1:1. Error bars are 95% confidence limits. Closed symbols refer to fitted results: EE (triangle), HE (circle), HH (square), and EH (diamond); open squares refer to summation results.

few percent bias (low) relative to the fully resolved case. The four fitted combinations result in a systematic trend for which the 10% valley ratios are slightly underestimated, with overestimation for the higher degrees of overlap. The order of overestimation is $EH > EE > HH > HE$, and the best accuracy is obtained with the HE combination. Precision degrades with increasing overlap in all cases.

The confidence limits plotted in Figure 7 are constructed from the standard deviations of the pooled replicates for each fit and, when calculated in this way, are not significantly different from the 0% valley. However, close inspection of the data reveals that most of the variability could be attributed to random variation from run to run, which could not be attributed to variation in signal or injection volume. Therefore, each of six differences in means among the four combinations was calculated for each replicate, and the resulting *mean differences* were tested as significantly different from zero. In every case, except one with particularly high variability, differences were found to be significant between combinations (95% confidence limits). This indicates that quantitative analysis using curve fitting is sensitive to choice of function. The four differences were calculated between the summation results and each function. Differences for all combinations except HE were statistically significant. The HE combination data at the 10 and 70% valleys were not statistically different from the summation results and were marginally significant ($P < 0.03$) at the 40% valley. These results indicate that quantitative information is best preserved by the HE combination under these conditions.

Mass 44 area ratios for fitting of the unequal abundances are presented in Figure 8. Figure 8a shows the area ratios resulting from 10:1 data set. As expected, the precision is substantially poorer than in the 1:1 case. However, mean differences in area ratio are more dramatic than in the 1:1 case, as many of the mean ratios are statistically different. As expected, most area differences (14 of 18) were statistically significant. The summation ratio is severely underestimated for both degrees of overlap for which there are data (10 and 40%). The 10% valley is the only overlap in which there are replicate summation data, and they are not statistically different from any of the fits.

Figure 8b presents the 1:10 data. Here all ratios are systematically shifted to lower values as a function of overlap and there is much better agreement among fits and summation. None of the areas resulting from these fits are significantly

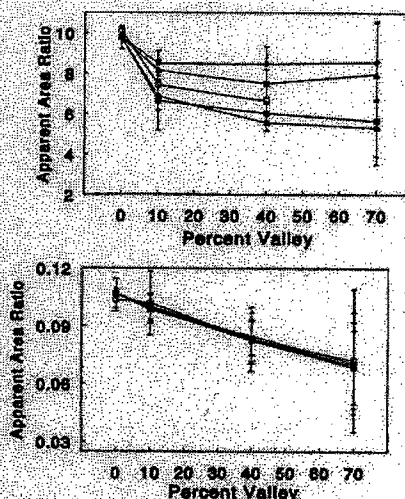


Figure 8. Area ratios for unequal abundance data calculated using the mass 44 signal: Me13:0/BHT of (a, top) 10:1 and (b, bottom) 1:10. The symbol key is given in the legend to figure 7.

different from the summation data. However, all but one fit is statistically different from one another at 10% valley. At the higher degrees of overlap only 1 of 12 comparisons was statistically significant.

These results indicate that choice of functions is important for trailing smaller peaks at all degrees of overlap; for leading small peaks the choice of functions is important only for low degrees of overlap. This difference in behavior in both isotope ratio and abundance calculations for the unequal abundance mixtures probably arises because of the asymmetric shape of the GCC/IRMS peaks considered here. In nearly all regards, the 1:10 mixture is better behaved than the 10:1 mixture, suggesting that the large tail in the latter case is not optimally modeled by these functions. Functions show a tendency to compensate for deficiencies in the modeling of the remaining peak. This was shown dramatically earlier and probably operates here on a subtle level. The overall greater effectiveness of the HE combination over the wide range of overlaps and abundance ratios may be due to the resistance of the H function to tail and the E function to front. The HH combination was also highly effective but was less effective than HE in some cases.

CONCLUSIONS

Overlapping peaks detected by conventional algorithms are systematically distorted in isotope ratio even for closely matched compounds, though high precision is maintained. Further, small trailing peaks can significantly affect the apparent isotope ratio of the major peak. Curve fitting is effective in restoring isotope ratios for overlap as high as 70% valley, and its effectiveness depends on the relative abundances and elution order as overall peak areas become more unequal. In none of the cases investigated did the fitted data result in poorer accuracy than the conventional summation method; the fitted data were either improved or not degraded. Our data indicate that the HVL/EMG or HVL/HVL combina-

tions provide the best fits of the functions considered in detail and are most robust and rapid for automated fitting of GCC/IRMS chromatograms. Small trailing peaks are the most difficult to model, apparently because they are obscured by the tail of the preceding peak. Small leading peaks are more easily modeled, and their minor effects on the major trailing peak can be effectively removed within the experimental error of these measurements. Finally, chromatographic peak shapes depend on specific chromatographic configuration and conditions, for which other functions may yield improved performance.

ACKNOWLEDGMENT

This work was supported by NIH Grant GM49209 and by the USDA—CSRS. K.J.G. acknowledges the support of NIH Training Grant DK07158.

Received for review October 6, 1993. Accepted January 27, 1994.*

* Abstract published in *Advance ACS Abstracts*, March 1, 1994.

On-line Recording of $^{13}\text{C}/^{12}\text{C}$ Ratios and Mass Spectra in one Gas Chromatographic Analysis

Wolfram Meier-Augenstein^{*1}

University Children's Clinic, Stable Isotope and GC-MS Laboratory, Im Neuenheimer Feld 150, D-69120 Heidelberg, Germany

Key Words:

Capillary GC
Isotope ratio MS
GC-on-line combustion
IRMS-ITD coupling
GC capillary eluate splitting
Organic acids

Summary

An isotope ratio mass spectrometer and an ion trap mass spectrometer have been coupled in parallel to one gas chromatograph. Using this arrangement both the $^{13}\text{C}/^{12}\text{C}$ ratio and the mass spectrum of a given compound can be measured simultaneously under identical gas chromatographic conditions. Although column connection fittings are employed to split the capillary eluate, chromatographic performance was barely impaired. Development of the system has been monitored by recording $\delta^{13}\text{C}$ values of the constituents of Grob test mixture no. 2. The accuracy and reliability of system performance has also been tested.

1 Introduction

Since the introduction of isotope ratio mass spectrometers (IRMS) coupled on-line to a gas chromatograph (GC) via a combustion interface (C), there has been an increasing interest in their analytical applications [1–5]. The merits of such systems are, however, somewhat limited because any statement about the isotopic composition of a given compound is exclusively based upon its gas chromatographic identification. When analyzing complex mixtures, such as plant extracts or physiological fluids, containing components present in an extremely wide range of concentrations, reliable measurement of isotopic composition is, furthermore, severely hampered by loss of column performance resulting in bad peak shape and insufficient separation. One solution to this problem is multidimensional GC-IRMS [6]. Another approach is to use thick-film capillary columns, which combine high resolution with good capacity [7].

All disciplines of analytical chemistry usually deal with mixtures of at least partly unknown composition. Not only unknown compounds of potential diagnostic value but also actually known compounds should and must be unambiguously identified. However accurate, the GC-C-IRMS technique only provides information on isotopic composition of a given compound. Identification of compounds relies solely upon retention indices. For unambiguous identification, repetition on another conventional GC-MS system is necessary, yet this solution is tainted with some uncertainty.

The logical step was to develop a system providing the means to obtain both mass spectrometric information and information about isotopic composition under identical gas chromatographic conditions, as described previously [8]. Maintaining the high

resolution achieved is absolutely essential for obtaining reliable measurements of isotopic enrichment. Owing to the unique design of the GC-C-IRMS, splitting of the GC eluate proved to be the most critical step. Difficulties encountered here most recently, prompted some modification in this respect.

2 Experimental

2.1 Instrumentation

The major components of the new system are a Varian model 3400 GC, a Delta S isotope ratio mass spectrometer (IRMS), a combustion interface, and a Magnum ion trap mass spectrometer (ITD); all, except for the GC, were designed and manufactured by Finnigan MAT. The ITD was chosen because its unique properties in respect of its vacuum and sample size requirements made it the most suitable candidate. The IRMS equipment, namely an oxidation oven and a reduction oven in the combustion interface, together with 2×3 Faraday cups for recording masses 44, 45, 46, and 28, 29, 30, respectively, enables the measurement of either ^{13}C or ^{15}N enrichment.

Uncoated deactivated fused silica capillaries of various length and internal diameter were chosen as connecting components in a way that in direct mode, i.e. with both back-flush valves closed, the split ratio was 20:1 between the combustion interface and ion trap outlet, respectively. This split ratio best meets the different needs of the two mass spectrometers. About 95 % of the GC eluate enters the combustion interface because the IRMS needs ca 1 nmol carbon in its ion source to ensure reliable results. About 5 % of the GC eluate enters the ITD because even in full scan mode sample amounts of 1 to 100 pmol are sufficient for this system.

To prevent solvent peaks entering the combustion oven, Matthews and Hayes [9] used a single solvent bypass valve linked to a vacuum pump. The state of the art Finnigan GC-C-IRMS system comes with a back-flush device which basically consists of two valves switchable in parallel. When open, in a direction opposite to that of normal carrier gas flow, helium is directed into the combustion furnace at about 2 ml/min. Part of this device is a Valco T-piece placed between column and combustion oven. One of the outlets is connected to one of the two back-flush valves which is open in back-flush mode; the other outlets are connected to the end of the capillary column and a fused silica capillary leading into the combustion oven, respectively.

¹ Present address: University of Dundee, Department of Anatomy and Physiology, Dundee DD1 4HN, UK.

To split the GC eluate, first a Valco cross-piece (Valco Instruments Europe, Schenkon, Switzerland) and later cross pieces manufactured by Gerstel (Gerstel GmbH, Mülheim, Germany) were employed. All the cross pieces have virtually no dead-volume but exhibit different properties in respect of leak tightness and thermal mass.

The connectors used for the coupling to the ITD are manufactured by Scientific Glass Engineering (SGE GmbH, Weiterstadt, Germany). The capillary coming from the cross piece, an additional helium make up capillary, and the capillary leading to the transfer line of the ITD were mounted in the connectors to form an open-split interface as previously described in detail [8]. The additional helium make-up is necessary because, in contrast to magnetic sector and conventional quadrupole MS which operate under high vacuum conditions, e.g. 10^{-6} mbar, an ITD requires a critical helium pressure, ca 10^{-2} mbar, in the ion trap for reliable operation.

2.2 Testing Conditions

All tests were performed under standardized conditions. Helium was used as carrier gas and its linear velocity was set to 28.6 cm/s. A 40 m \times 0.32 mm glass capillary column coated with a 0.32 μm film of OV-1701-OH and a 30 m \times 0.25 mm fused silica capillary column coated with a 1 μm film of DB-5 (J&W) were used. Sample volumes were 1.2 and 2.1 μl for the OV-1701 column and DB-5 columns, respectively. The split ratio was set to 1:20. The oven temperature was held at 40 $^{\circ}\text{C}$ for 5 min and then programmed to 280 $^{\circ}\text{C}$ at 4 $^{\circ}\text{C}/\text{min}$. Column test mixture 2 according to Grob (Fluka, Neu-Ulm, Germany) was used throughout all experiments.

A dichloromethane solution (3 nmol/ml) of a mixture of authentic methyl esters of organic acids (*RS*)-methyl lactate, methyl (*R*)-3-hydroxybutyrate, dimethyl glutarate, methyl phenylacetate, and methyl laurate (Fluka) was analyzed to check the system for potential influence of GC eluate splitting on measurement of isotopic composition compared with direct mode operation using a T-piece.

The oxidation and reduction ovens of the combustion interface of the IRMS were operated at 940 and 600 $^{\circ}\text{C}$, respectively. In back-flush mode, helium was directed into the combustion furnace at 2 ml/min to prevent, e.g., solvent peaks entering the combustion oven and thereby prematurely exhausting its oxidation capacity.

3 Results and Discussion

3.1 Valco Cross-Piece

In first attempts to couple an ITD in parallel to an IRMS a Valco cross-piece was substituted for the Valco T-piece [8]. Initial Grob tests performed using temperature gradients of 1 $^{\circ}\text{C}/\text{min}$ and less [10] looked rather promising. With temperature gradients of 2 $^{\circ}\text{C}/\text{min}$ and more, however, considerable loss of chromatographic performance was observed (Figure 1). Owing to drastic peak broadening none of the polar compounds in the test mixture could be detected by the IRMS. Having passed the combustion furnace their corresponding carbon dioxide peaks disappeared in the m/z 44 baseline.

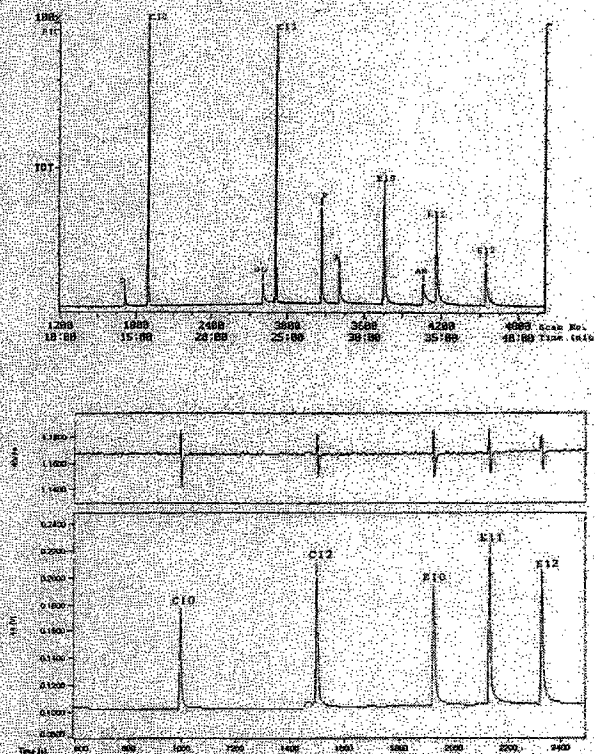


Figure 1. GC analysis of the test mixture on OV-1701-OH, using a Valco cross-piece as capillary eluate splitter. (A) Reconstructed ion chromatogram (RIC) obtained on the ITD; (B) ion current of m/z 44 (carbon dioxide) simultaneously recorded on the IRMS with calculated 45/44 ratio-trace; D, 2,3-butanediol; C10, *n*-decane; C12, *n*-dodecane; P, 2,6-dimethylphenol; S, 2-ethylhexanoic acid; A, 2,6-dimethylaniline; Am, diethylohexylamine; E0-E12, methyl esters of decanoic, undecanoic, and dodecanoic acids.

Furthermore, in contrast with the claimed leak tightness up to temperatures of 300 $^{\circ}\text{C}$, the Valco cross-piece started to leak even after one run ending at e.g. 280 $^{\circ}\text{C}$. More precisely, it was leak tight up to 280 $^{\circ}\text{C}$ but after cooling to 40 $^{\circ}\text{C}$ leaks were detected at all four connecting screws. The formerly tightened screws had worked themselves loose, possibly as a result of thermal expansion of the solid metal body of the cross-piece.

This behavior substantiated the idea that the high mass (15 g) of this cross-piece was also the reason for its observed influence on chromatographic performance. Owing to the extreme peak broadening of the polar constituents of the test mixture and the peak tailing of the fatty acid methyl esters, it was believed that the Valco cross-piece acted as cold spot.

When extreme peak broadening and peak tailing were first observed all obvious sources had been checked. Capillary columns were double-checked for chromatographic performance on a GC (HRGC 5300 Mega Series, Carlo Erba) and a GC-MS (Varian GC 3400-TSQ 70, Finnigan). Injector inserts and all connectors were deactivated prior to use by treatment with 1 ml of a 1:1 (v/v) mixture of dimethylchlorosilane and hexamethyldisilazane in *n*-heptane (50 ml). In the meantime fused silica connecting cap-

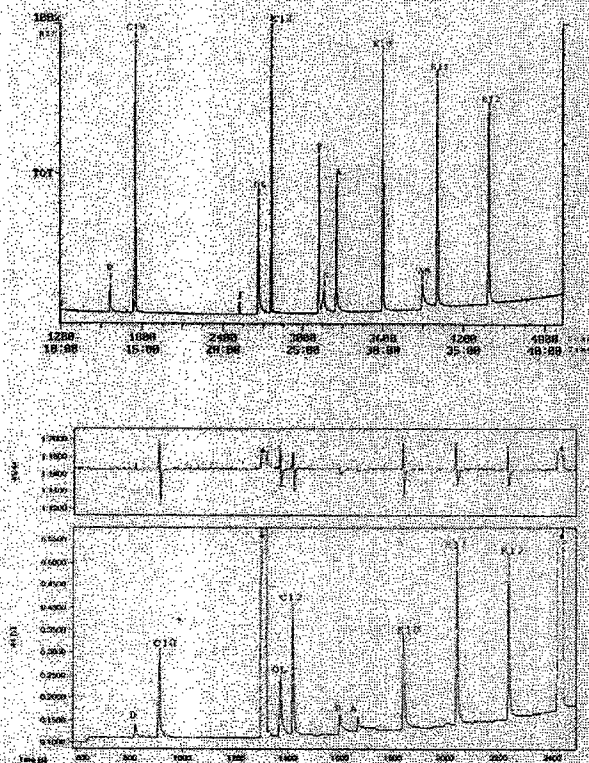


Figure 2. GC analysis of the test mixture on OV-1701-OH, using a standard Gerstel cross-piece as capillary eluate splitter. (A) RIC of the ITD. (B) ion chromatogram of the IRMS. Asterisks indicate reference gas peaks. Peak identification as for Figure 1.

illaries deactivated with a 1 nm coating of OV-1701-OH were used [11].

3.2 Standard Gerstel Cross-Piece

Prompted by the aforementioned observations an alternative to the Valco cross-piece had to be found. As a result of good experience with Gerstel 3D capillary column connectors in respect of leak tightness, low dead volume, and influence on chromatographic performance a Gerstel 3D cross-piece seemed suitable.

The standard Gerstel cross-piece weighs 5 g. Figure 2 shows a GC chromatogram of the test mixture obtained after substitution of the Gerstel cross-piece for the Valco cross-piece. Although the test chromatogram now showed all the polar compounds, there was still room for improvement. S and Am were, for instance, still relatively small and broad and could not, therefore, be detected with the IRMS.

In contrast to the Valco cross-piece, the Gerstel cross-piece stayed leak tight even after repeated analyses ending at 300 °C. No difference in nitrogen, argon, and water levels between separate analyses could be detected on either mass spectrometer.

3.3 Custom-Made Gerstel Cross-Piece

The next logical step was to design a light-weight cross-piece by literally cutting down on material without losing stability. To meet this requirement, Gerstel produced a custom-made cross-piece weighing 3.18 g only.

Figure 3A shows that the resulting test chromatogram bears virtually no difference from a test chromatogram obtained when the capillary column was installed directly into the transfer line of the ITD (Figure 3B). When these two reconstructed ion chromatograms (RIC) are compared more closely, slight peak tailing can be observed in Figure 3A. This is certainly caused to a certain extent by the cross-piece. Most of this effect is, however, a consequence of the manner in which the chromatograms are depicted, i.e. by proportional amplification of ubiquitous peak tailing as both RICs are normalized to the most intense peak. In Figure 3B the ion current of the baseline is ca 8700 whereas in Figure 3A it is ca 1200. So, in Figure 3B the ubiquitous peak tailing is pushed into the base line. When the peak tail ion currents were compared relative to the corresponding baseline ion currents it was found that peak tailing in Figure 3A was only 30 % greater than the ubiquitous peak tailing.

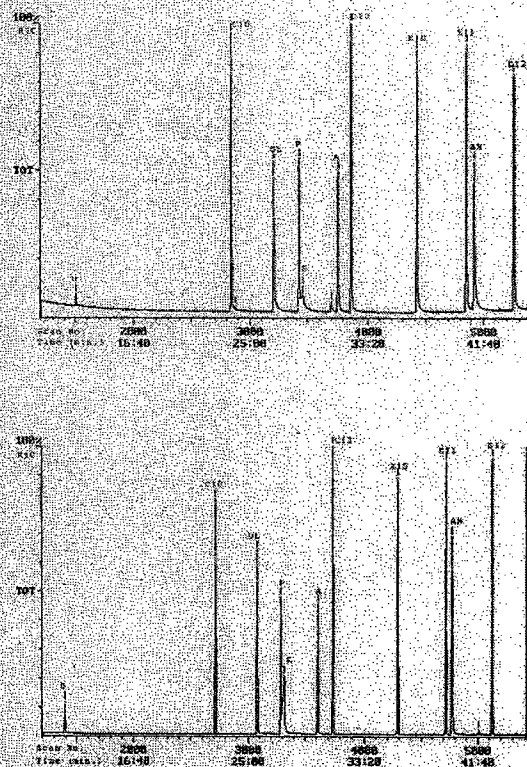


Figure 3. GC analysis (RIC) of the test mixtures on DB-5, using (A) the light-weight Gerstel cross-piece as capillary eluate splitter and (B) when the column end was installed directly into the ITD transfer line. Peak identification as for Figure 1.

When leak tightness was checked, the customized cross-piece showed the same properties as the standard cross-piece. This cross-piece has since been used for more than 200 analyses ending at 300 °C without the need for retightening or changing of a seal.

3.4 System Performance and Reliability

Obviously, reliable GC-C-IRMS requires good GC performance because both precision and accuracy of IRMS measurements are limited by chromatographic resolution. Table 1 lists $\delta^{13}\text{C}$ values of the constituents of Grob test mixture 2; these clearly demonstrate the influence of this on accuracy. Differences in peak shape and, hence, in peak integral width from one analysis to another, yield $\delta^{13}\text{C}$ values showing standard deviations of nearly 0.1 %. Although accuracy and precision improve with as the influence of the cross-pieces on GC performance is reduced, no significant shift in $\delta^{13}\text{C}$ values can be observed.

To check the system for reliability, i.e. for potential isotopic fractionation as a result of GC eluate splitting, a mixture of methyl esters of five organic acids was analyzed on the original GC-C-IRMS (direct coupling *via* T-piece) and on the hybrid system with GC eluate splitting *via* the light-weight cross-piece. This mixture was chosen because comparable chromatographic behavior on both systems could be expected for all compounds except for the two hydroxy acids which showed extreme peak broadening on the original GC-C-IRMS system. Comparison of $\delta^{13}\text{C}$ values obtained on the original and hybrid systems (Table 2) revealed no evidence of isotopic fractionation.

4 Conclusion

Coupling an IRMS and an ITD in parallel to one GC by means of a light-weight cross-piece and a home-made open-split connector resulted in a hybrid GC-MS system which makes it possible both to determine isotopic composition and to obtain structural information by recording mass spectra of any given compound simultaneously in one GC analysis with virtually no loss of chromatographic performance. Peak tailing in addition to ubiquitous peak tailing amounts to approximately 30 % at the most. Shifting of $\delta^{13}\text{C}$ values as a result of isotopic fractionation was never observed.

This hybrid system combines the sensitivity of an IRMS for isotopic enrichment and the trace sensitivity of an ITD in one system, opens a new line of applications for isotope ratio mass spectrometry, and improves the conclusiveness of existing applications. All areas of research and applied analytical chemistry, e.g. food quality control, nutritional sciences, environmental monitoring, toxicology, and biomedicine will profit from the potential of this system.

Acknowledgments

This work was supported in part by the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany) Grant No. AZ 3772-125-348. The author wishes to thank Professor B. V. Burger (Department of Chemistry, University of Stellenbosch, RSA) for making the OV-1701-OH column, and Professor D. Rating (University Children's Clinic Heidelberg) for his support during this work.

Table 1. $\delta^{13}\text{C}_{\text{PDB}}$ values [‰] of the constituents of Grob test mixture 2.^{a)}

| Constituent ^{b)} | Valco standard X-piece | | | Gerstel standard X-piece | | | Gerstel light-weight X-piece | | |
|---------------------------|------------------------------------|------|---|------------------------------------|------|---|------------------------------------|------|---|
| | $\delta^{13}\text{C}_{\text{PDB}}$ | SD | n | $\delta^{13}\text{C}_{\text{PDB}}$ | SD | n | $\delta^{13}\text{C}_{\text{PDB}}$ | SD | n |
| D | n.d. | — | — | -24.47 | 0.13 | 5 | n.d. | — | — |
| C ₁₀ | -42.26 | 0.71 | 5 | -41.04 | 0.06 | 5 | -41.72 | 0.21 | 5 |
| Ol | -32.44 | 0.98 | 5 | -30.36 | 0.27 | 5 | -29.32 | 0.25 | 5 |
| C ₁₂ | -26.87 | 0.52 | 5 | -25.79 | 0.29 | 5 | -25.21 | 0.26 | 5 |
| P | n.d. | — | — | -36.25 | 0.49 | 5 | -35.41 | 0.26 | 5 |
| S | n.d. | — | — | n.d. | — | — | -16.84 | 0.24 | 5 |
| A | n.d. | — | — | -31.19 | 0.52 | 5 | -31.48 | 0.27 | 5 |
| E ₁₀ | -30.01 | 0.96 | 5 | -29.56 | 0.28 | 5 | -29.34 | 0.23 | 5 |
| Am | n.d. | — | — | n.d. | — | — | -25.14 | 0.24 | 5 |
| E ₁₁ | -27.16 | 0.49 | 5 | -26.72 | 0.29 | 5 | -26.27 | 0.25 | 5 |
| E ₁₂ | -30.54 | 0.64 | 5 | -30.06 | 0.34 | 5 | -29.59 | 0.23 | 5 |

^{a)} The GC analyses were performed on the OV-1701-OH column.

^{b)} The abbreviations are explained in Figure 2.

Table 2. $\delta^{13}\text{C}_{\text{PDB}}$ values [‰] of commercially available methyl esters of organic acids.^{a)}

| Methyl ester of: | Valco standard X-piece | | | Gerstel light-weight X-piece | | |
|---------------------------|------------------------------------|------|---|------------------------------------|------|---|
| | $\delta^{13}\text{C}_{\text{PDB}}$ | SD | n | $\delta^{13}\text{C}_{\text{PDB}}$ | SD | n |
| (R,S) Lactic acid | n.d. | — | — | -30.28 | 0.23 | 5 |
| (R)-3-Hydroxybutyric acid | -15.17 | 0.76 | 5 | -15.42 | 0.03 | 5 |
| Glutaric acid | -26.64 | 0.67 | 5 | -26.34 | 0.13 | 5 |
| Phenylacetic acid | -28.18 | 0.71 | 5 | -27.95 | 0.15 | 5 |
| Lauric acid | -27.86 | 0.59 | 5 | -28.27 | 0.12 | 5 |

^{a)} The GC analyses were performed using the OV-1701-OH column.

References

- [1] A. Mosandl, U. Hener, H.-G. Schmitt, and M. Rautenschlein, *J. High Resol. Chromatogr.* 13 (1990) 528.
- [2] M. Woerner and P. Schriber, *Z. Lebensm.-Unters. Forsch.* 190 (1990) 425.
- [3] A. Bernreuther, J. Koziel, P. Brunerie, G. Krammer, N. Christoph, and P. Schriber, *Z. Lebensm.-Unters. Forsch.* 191 (1990) 299.
- [4] K.J. Goodman and J.T. Brenna, *Anal. Chem.* 64 (1992) 1088.
- [5] Y. Khalifah, S. Normand, S. Tissot, C. Pachiaudi, M. Beylot, and J.P. Riou, *Biol. Mass Spectrom.* 22 (1993) 707.
- [6] S. Nitz, B. Weinreich, and P. Driewert, *J. High Resol. Chromatogr.* 15 (1992) 387.
- [7] W. Meier-Augenstein, G.F. Hoffmann, B. Holmes, J.L. Jones, W.L. Nyhan, and L. Sweetman, *J. Chromatogr. Biomed. Appl.* 615 (1993) 127.
- [8] W. Meier-Augenstein, W. Brand, G.F. Hoffmann, and D. Rating, *Biol. Mass Spectrom.* 23 (1994) 376.
- [9] D.E. Mathews and J.M. Hayes, *Anal. Chem.* 50 (1978) 1465.
- [10] K. Grob, *Making and Manipulating Capillary Columns for Gas Chromatography*, Hüthig, Heidelberg, Germany (1986) 193.
- [11] G.R. van der Hoff, P. van Zoanen, and K. Grob, *J. High Resol. Chromatogr.* 17 (1994) 37.

Ms received: June 24, 1994

Accepted: November 8, 1994

SHORT COMMUNICATION

Bridging the Information Gap Between Isotope Ratio Mass Spectrometry and Conventional Mass Spectrometry

Wolfram Meier-Augenstein†

University Children's Hospital, Stable-Isotope- and GC-MS-Laboratory, Im Neuenheimer Feld 150, D-69120 Heidelberg, Germany

Willi Brand

Finnigan MAT GmbH, Barkhausenstr. 2, D-28197 Bremen, Germany

Georg F. Hoffmann

University Children's Hospital, Department of Metabolic Disorders, Im Neuenheimer Feld 150, D-69120 Heidelberg, Germany

Dietz Rating

University Children's Hospital, Department of Child Neurology, Im Neuenheimer Feld 150, D-69120 Heidelberg, Germany

An isotope ratio mass spectrometer and an ion trap mass spectrometer were coupled in parallel to one gas chromatograph. This system provides the means to obtain information about both chemical nature and isotopic composition of a given compound under identical gas chromatographic conditions.

INTRODUCTION

Since the introduction of isotope ratio mass spectrometers (IRMS), on-line coupled to a gas chromatograph (GC) via a combustion interface (C), there has been increasing interest in its various analytical applications.¹⁻⁵ The merits of this system, however, are somewhat limited because the statement about the isotopic composition of a given compound is exclusively based upon its unambiguous gas chromatographic identification.

This demand can only be met in cases of injection of either single compounds or not too complex mixtures. Otherwise, the identification of single peaks becomes biased, as it relies solely on comparison of retention indices as well as the assumption that each peak is caused by one compound only.

In all disciplines of analytical chemistry one usually deals with mixtures of at least partly unknown composition. Not only unknown compounds of potential diagnostic value but also actually known compounds should and must be unambiguously identified. Additionally, sometimes only a very limited amount of material to be investigated is available. The GC/C/IRMS technique requires at least 1 nmol carbon in the ion source. Therefore, repetition of the analysis on another conventional GC/MS system may not be possible. Furthermore, this is not an acceptable solution of the problem. Slight changes in carrier gas flow, non-identical GC oven conditions, replacement of columns and variation in the concentration range of the sample, to name but a few, are typical sources of error that strongly influence GC performance and hence hamper reproducibility and reliability of an analysis.

† Author to whom correspondence should be addressed.

The logical step is to devise a system providing the possibility of obtaining both mass spectrometric information and knowledge about isotopic composition under identical gas chromatographic conditions. Here, we wish to present such a system where an IRMS and an ion trap mass spectrometer are coupled in parallel to one GC.

MATERIALS

The major components that comprise the new system are a Varian GC, model 3400, a Delta S isotope ratio mass spectrometer, a combustion interface and a Magnum ion trap mass spectrometer, all manufactured and designed by Finnigan MAT except for the GC. The ion trap mass spectrometer was chosen because its unique properties with respect to its vacuum and sample size requirements made it the most suitable candidate.

For connecting those components, uncoated deactivated fused-silica capillaries of various internal diameters were used. The cross-piece that splits the effluent coming from the GC column is made by Valco Instruments Co. Inc. This cross-piece has virtually no dead volume and its fittings are leaktight. The connectors used for the coupling to the ion trap mass spectrometer are made by Scientific Glass Engineering Pty Ltd (S.G.E.).

RESULTS AND DISCUSSION

In order to prevent solvent peaks entering the combustion oven, and thereby prematurely exhausting the oxidation catalyst, Matthews and Hayes⁶ used one solvent bypass valve that was linked to a vacuum

pump. The state-of-the-art Finnigan GC/C/IRMS system comes with a backflush device that basically consists of two valves switchable in parallel. When open, in the opposite direction towards the carrier gas flow, helium is directed into the combustion furnace at about 2 ml min^{-1} (Fig. 1). Part of this device is a Valco cross-piece placed between column and combustion oven (Fig. 2). One of the two remaining outlets is con-

nected to one of the two backflush valves that is open in backflush mode; the other outlet, connected to a fused-silica capillary, is permanently open. To this outlet the ion trap mass spectrometer was linked.

The internal diameters of the capillaries involved are chosen in such a way that in straight mode, i.e. both backflush valves closed, the split ratio combustion interface to ion trap outlet is 9:1, as this split ratio meets

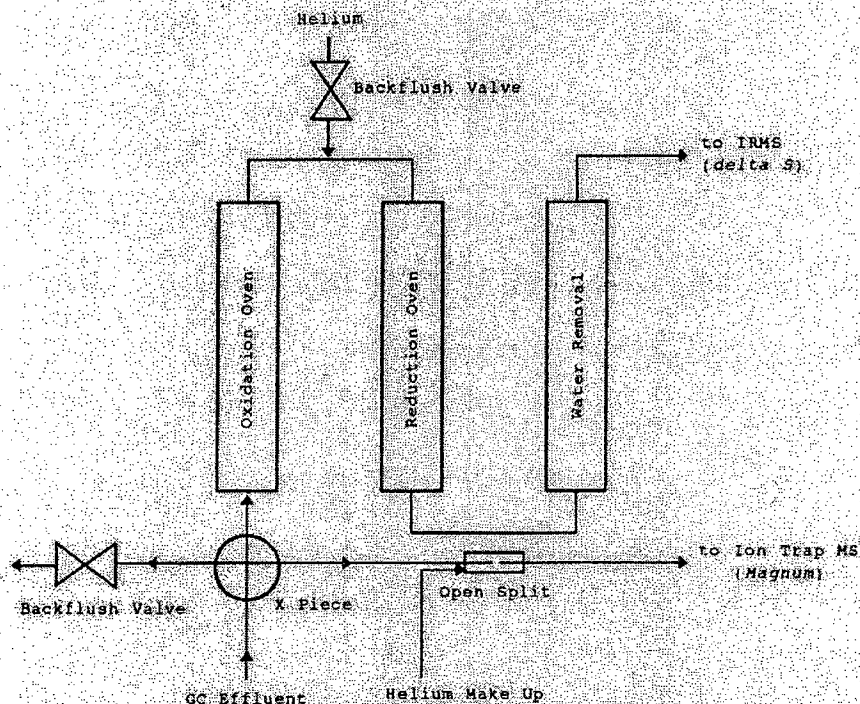


Figure 1. Schematic layout of the combustion interface with backflush device and coupling to the isotope ratio mass spectrometer and the ion trap mass spectrometer.

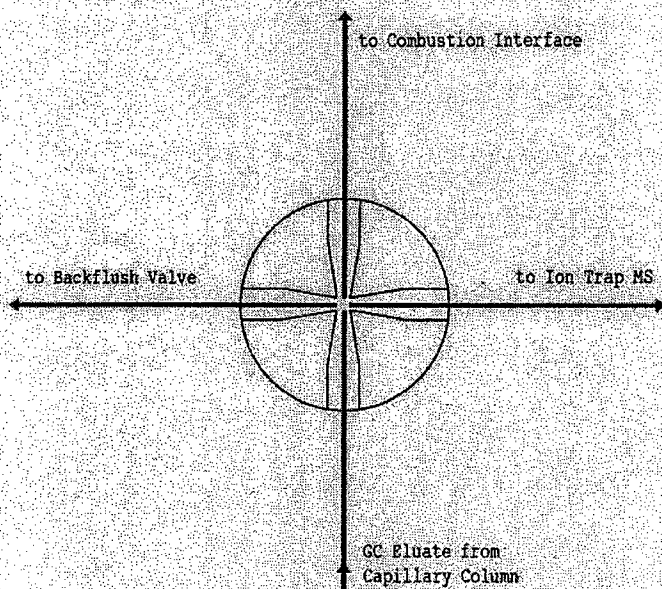


Figure 2. Cross-section through the Valco cross-piece that serves as split. Note that internal diameters of capillaries are not identical.

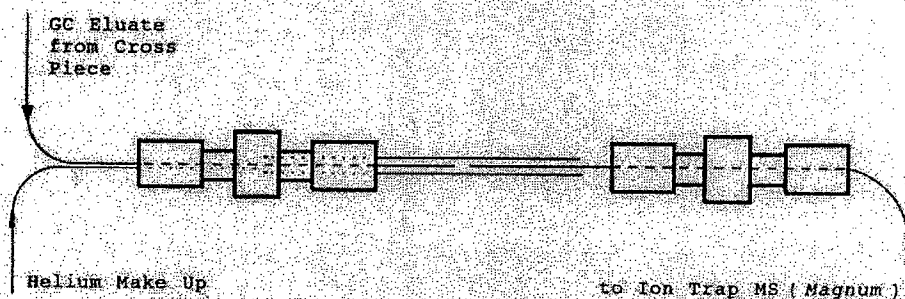


Figure 3. Sketch of the open split connector to the ion trap mass spectrometer.

best the different needs of the two mass spectrometers. About 90% of the GC effluent enters the combustion interface because the IRMS needs an amount of about 1 nmol carbon in its ion source to ensure reliable results. About 10% of the GC effluent enters the ion trap mass spectrometry, because for this system sample amounts of 1–100 pmol are sufficient.

Whereas the outlet leading to the combustion interface is directly connected to the combustion furnace, the capillary leading to the ion trap mass spectrometer is integrated into an open split⁷ that basically consists of two SGE linear connectors (Fig. 3). In contrast to magnetic sector and conventional quadrupole mass spectrometers which operate under high vacuum conditions, an ion trap mass spectrometer requires a certain helium pressure, i.e. 10^{-3} mbar, in the ion trap to operate reliably. For that, an additional helium make-up is led to one of the aforementioned connectors by a second capillary. Both capillaries are mounted into the left side of the first connector by a two-hole ferrule. The carrier gas capillary is completely led through this connector and finally ends in a fused-silica capillary that is mounted into the right side of the connector. The make-up capillary, however, is only introduced halfway into the connector, thus forming a constant helium cushion around the carrier gas capillary and inside the surrounding capillary. From its open end, the mass spectrometer interface capillary, mounted straight through a second SGE linear connector, is introduced to meet the carrier gas capillary in the middle of the surrounding capillary, leaving a gap of less than 1 mm between them.

In backflush mode the amount of sample material entering the ion trap mass spectrometer is considerably smaller, however. As the backflush is generally used to cut out solvent peaks, this loss of information can be

tolerated. Usually, acquisition of mass spectroscopic data is started after elution of the solvent peak anyway.

None of the aforementioned connecting parts, i.e. cross-piece and SGE connectors, causes detectable loss in chromatographic performance due to cold spots. The system has been tested using Grob-test mixture no. 2 on three capillary columns coated with stationary phases of different polarity and different film thickness, e.g. 0.17 μ m HP Ultra 1, 1.0 μ m J & W DB-5, 0.3 μ m OV 1701-OH. In straight mode, reconstructed ion currents for constituents of 1.5 μ l Grob-test mixture No. 2, injected in split mode 1 to 10, are in the range of 30 000–260 000.

CONCLUSION

We think the system will open a new line of applications for isotope ratio mass spectrometry and will improve the conclusiveness of existing applications. Sample preparation up to now involving several steps, each bearing the risk of isotopic fractionation,⁸ can be simplified. Peaks, unexpectedly showing isotopic enrichment, e.g. in tracer studies of biochemical pathways, can be identified by on-line recording their mass spectra.

In short, all the areas of research and applied analytical chemistry, where stable isotope labelled compounds or detection of small changes in isotopic composition are involved, will profit from the potential of such a system.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany) HO 966/3-1 and the University Children's Hospital, Heidelberg, is gratefully acknowledged.

REFERENCES

1. A. Mosandl, U. Henner, H.-G. Schmitt and M. Rautenschleim, *J. High Resolut. Chromatogr.* **13**, 528 (1990).
2. M. Woerner and P. Schreier, *Z. Lebensm.-Unters. Forsch.* **190**, 425 (1990).
3. A. Bernreuther, J. Koziet, P. Bruneris, G. Krammer, N. Christoph and P. Schreier, *Z. Lebensm.-Unters. Forsch.* **191**, 299 (1990).
4. K. J. Goodman and J. T. Brenna, *Anal. Chem.* **64**, 1088 (1992).
5. Y. Khalfallah, S. Normand, S. Tissot, C. Pachiaudi, M. Beylot and J. P. Riou, *Biol. Mass Spectrom.* **22**, 707 (1993).
6. D. E. Matthews and J. M. Hayes, *Anal. Chem.* **50**, 1465 (1978).
7. D. Henneberg, U. Henrichs and G. Schomburg, *Chromatographia* **8**, 449 (1975).
8. R. Braunsdorf, U. Henner, S. Stein and A. Mosandl, *Z. Lebensm.-Unters. Forsch.* **197**, 137 (1993).

High-precision gas chromatography–combustion isotope ratio mass spectrometry at low signal levels

Keith J. Goodman, J. Thomas Brenna*

Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853, USA

First received 30 August 1994; revised manuscript received 7 October 1994

Abstract

Precision and accuracy of gas chromatography–combustion isotope ratio mass spectrometry are investigated for sample levels down to about 5 pmol C in fatty acid methyl ester mixtures spanning 1000-fold in concentration. Precision and accuracy of isotope ratios diverge rapidly for conventional summation methods, and become unusable below 30 pmol material on column. At lower levels, mean isotope ratios were statistically different from reference values indicating bias as well as poor precision. In contrast, curve fitting, using the exponentially modified Gaussian line shape, gives improved precision for most peaks and useful results down to 3 pmol. The curve-fitting algorithm was also less sensitive to signal integration time than the summation method. These data indicate that curve fitting may be the method of choice for integration of noisy data when high-precision isotope ratios are desired.

1. Introduction

High-precision gas [1,2] (or liquid [3,4]) chromatography–combustion isotope ratio mass spectrometry (x-C–C-IR-MS) for analysis of C or N isotopes [5,6] is gaining importance across many areas of natural science [7]. These techniques facilitate the analysis of individual compounds at very small sample sizes, with precision levels approaching that of conventional dual inlet [8]. Most applications take advantage of these improved parameters, as well as the capability to rapidly obtain high-precision compound-specific results without cumbersome chemical separation prior to analysis (e.g. Refs. [9–11]). However, a wide variety of applications require the analysis of complex mixtures, inevitably involving over-

lapping peaks. We previously reported that the conventional summation integration algorithm produces systematic bias at degrees of overlap as low as 10% valley, even for compounds of well matched isotope ratio (e.g. $\Delta\delta^{13}\text{C} < 0.5$) [12]. Most notably, this bias is observed while high precision is maintained. Further, isotope ratios for minor components (<10 ng) are seldom calculated because of overlaps and the well known difficulties in defining peak start/stop in low signal-to-noise ratio (S/N) cases. This is true even though high-precision results ($\delta^{13}\text{C} < 0.5$) can be achieved for sub-ng sample sizes under ideal circumstances [8].

We have recently shown that curve fitting can be effective in recovering accurate isotope ratios from severely overlapping peaks while preserving high precision [12]. Curve-fitting methods are known to be more resilient to low S/N than

* Corresponding author.

summation algorithms, where peak definition is most difficult [13–15]. Analysis of low levels by GC–C-IR-MS is of particular interest for tracer studies where precision as poor as $\delta^{13}\text{C} < 100$ is useful for detecting enriched metabolites [2]. We report here a systematic study comparing curve fitting to conventional summation for well-resolved small sample injections over four orders of magnitude in single GC–C-IR-MS chromatograms.

2. Experimental

2.1. Instrumentation

A Varian 3400 GC system interfaced to a Finnigan MAT 252 high-sensitivity gas isotope ratio mass spectrometer via a combustion interface was used for this study. The instrument was operated at an accelerating potential of 8 kV and a source pressure of $2 \cdot 10^{-6}$ Torr (1 Torr = 133.322 Pa). Briefly, the effluent of the capillary column is directed to a combustion furnace held at 850°C and loaded with CuO as a source of O_2 . Combustion products are dried and pass through an open split prior to entrance to the IR-MS ion source. CO_2 is monitored continuously at m/z 44, 45 and 46 with three dedicated faraday cups and associated electronics. Asymmetric peak shapes are commonly observed because of the numerous connections and changes in capillary diameter between the GC column and the mass spectrometer. Details of the system can be found elsewhere [2].

A fatty acid methyl ester (FAME) standard mixture was separated on a J & W (Folsom, CA, USA) DB-23 capillary column, (30 m \times 0.32 mm I.D., 0.25 μm film). Carrier flow through the GC column was 45 cm/s He (99.999 + %), with inline O_2 and H_2O traps. Fatty acid standards were obtained from Sigma (St. Louis, MO, USA) and were 99 + % pure by GC analysis. Hexane (Fisher Optima grade) was used whenever solvent was necessary, and samples were stored in glass vials with Teflon-lined caps. The test mixture was composed of methyl pentadecanoate (Me15:0), methyl heptadecanoate

(Me17:0), methyl octadecanoate (Me18:0) and methyl heneicosanoate (Me21:0) at concentrations of about 0.16, 1.6, 16 and 160 ng/ μl , respectively. Injections of 0.5 μl of this mixture were made in splitless injection mode. A second, equimolar mixture of these compounds (0.2 $\mu\text{g}/\mu\text{l}$ each) was prepared and analyzed separately to establish reference isotope values for each compound.

The vendor-supplied data acquisition system collects a signal from each mass channel simultaneously for adjustable integration times. At the conclusion of an integration period, a counter is queried, zeroed, and restarted, giving a >99% duty cycle. Any particular integration time is a tradeoff between noise per data point and faithful reproduction of lineshape. Longer integration times improve S/N for each data point, but distort lineshape. This distortion can be important for accurate curve fits, as well as for precise definition of peak start/stop for summation. The test mixture was analyzed using integration times of 0.25, 0.125 or 0.0625 s to investigate the effect of this parameter on summation and on curve-fitting peak integration approaches. Four or five replicates were made at each integration time.

2.2. Data processing

The vendor-provided software "ISODAT" (Finnigan MAT, Bremen, Germany) was used to process the data by the summation method. A review of this software has been published recently [16]. Slope sensitivity (SS) for peak start/stop definition is set by the user. A rolling five-point linear least squares fit is applied to the chromatogram, with peak start defined when the slope exceeds the SS; stops are defined as the point beyond the peak where the slope falls below the absolute value of the SS. SS settings chosen to detect strong peaks and reject spurious noise-related peaks do not satisfactorily detect very small peaks, while settings used to detect small peaks are overly sensitive to noise. Therefore, two separate settings for slope sensitivity were compared, SS = 0.8 mV/s for higher level detection which has performed satisfactorily in

routine applications in our laboratory over the last three years, and $SS=0.1$ mV/s for low detection. Other workers report $SS=1$ mV/s as satisfactory for routine analyses [8].

The commercial program Peakfit (Jandel Scientific) was used for all curve fitting. Previously, we evaluated several functions for use with GC-C-IR-MS peaks [12]. The well-known exponentially modified Gaussian (EMG) function, constructed as the mathematical convolution of the Gaussian and exponential functions [15], accurately reproduces the line shapes as long as the column is not overloaded and appears to work particularly well for low signal levels. It was used exclusively in this work. Each set of four or five chromatograms for each integration time were processed with the $SS=0.8$, $SS=0.1$, and curve-fit methods.

As in previous work, we calculate high-precision isotope ratios according to the following:

$$\delta 45 = \left(\frac{R_{SPL} - R_{PDB}}{R_{PDB}} \right) \times 1000 \quad (1)$$

where R_x is the ratio of integrated areas for the m/z 45 to 44 channels, SPL and PDB refer to the sample and the international standard Pee Dee Belemnite, respectively, international standard with $R_{PDB} = 0.0112372$. This expression does not take into account the contribution of $^{12}C^{16}O^{17}O$ to the m/z 45 signal, which is small and in the case of GC-C-IR-MS is nearly constant. Areas are obtained by either fitting the m/z 44 and 45 channels or using the summation methods. No outliers were excluded from this data set.

Tests of statistical differences of means were performed using the t -distribution at the 95% confidence level [17].

3. Results and discussion

A representative chromatogram is presented in Fig. 1, and plotted in semilog format so that the four orders of magnitude peak intensities and background noise levels are discernable. Peak intensities above baseline range from about 2 mV for the smallest, earliest eluting peak to a high of

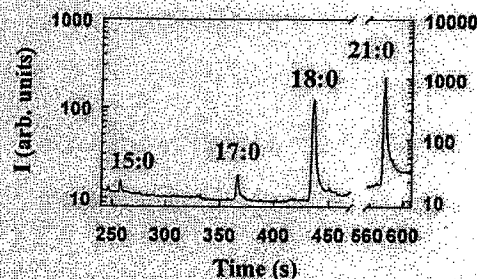


Fig. 1. GC-C-IR-MS chromatogram of the FAME test mixture plotted in semilog format.

500 mV for the most abundant but broader, latest eluting peak. There is a significant amount of chemical noise near the intensity of the smallest peak, which corresponds to 0.1% of the largest peak, Me21:0. As all FAMES in the mixture are 99+ % pure, contaminants at this level entering the test mixture with Me21:0 are expected. Signal outputted in volts by the data system was converted to current, and peak area in nA s was calculated and used for comparison.

Deviations from reference values in $\delta 45$ units (%) are presented in Figs. 2–4 for the individual determinations of FAMES at the three integration times investigated. The abscissa represents signal area and is approximately logarithmic, although points have been offset horizontally for illustrative purposes. Both summation methods diverge more rapidly than the fitted data as sample size decreases for all integration times. Performance of the summation methods degrades significantly at 63 ms integration time compared with the 125 and 250 ms case. In contrast, the curve-fit performance did not degrade significantly at the lower integration time. At 200 and 2000 nA s, precisions were best at 63 ms integration time, while the lower signal levels gave slightly better results at 250 ms integration time.

Precisions for the higher SS are superior to those for the lower SS at all levels. At the lowest signal level investigated, the $SS=0.8$ summation method failed to detect peaks reliably while the $SS=0.1$ method produced highly inaccurate and imprecise ratios. The summation results are also seen to deviate substantially from the reference

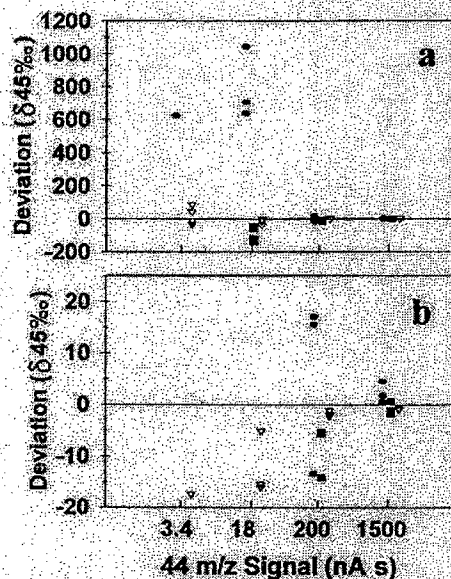


Fig. 2. $\delta 45$ deviations from reference values for 63 ms integration rate using the three different integration methods: \blacksquare = SS 0.8 mV/s; \bullet = SS 0.1 mV/s; ∇ = curve fit. Points for SS = 0.8 mV/s and fitted data are offset their actual signal level for illustrative purposes. Data of a is plotted in b with expanded ordinate.

levels. This observation is consistent with our previous report, and may also explain inaccuracy observed for highly enriched material at low sample levels [18]. In fact, S.D.s improve with shorter integration time for the strongest signal level.

Summary data of $\delta 45$ values are presented in Table 1, tabulated using average signal levels (in nA s), along with reference values from the equimolar mixture. Precisions (S.D.) for the reference values were all $\delta^{13}\text{C} < 0.4$ which are consistent with our routine measurements under nearly ideal GC-C-IR-MS conditions. The SS = 0.8 method detected only one peak at the lowest level across all integration times. Precisions and accuracy for the curve-fit data are superior for all but one cell.

Accuracy was assessed by statistically testing mean deviations for equivalence with zero. All of the 20 nA s deviations for the SS = 0.8 method were significantly different from zero, indicating systematic shifts in measured isotope ratio

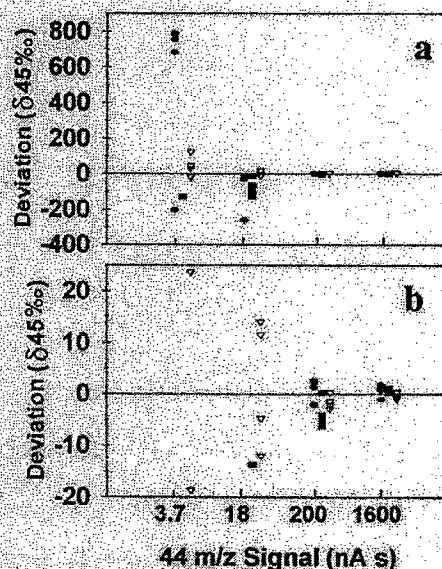


Fig. 3. $\delta 45$ deviations from reference values for 125 ms integration rate. Symbols as in Fig. 2.

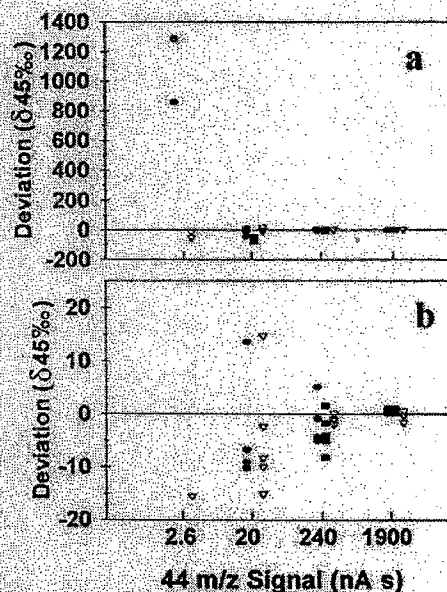


Fig. 4. $\delta 45$ deviations from reference values for 250 ms integration rate. Symbols as in Fig. 2.

Table 1
 845 (Mean \pm standard deviation)

| | Average area (nA s) | 845 | | | |
|---------------|---------------------|---------------------|--|---------------------|-------------------|
| | | 63 ms | 125 ms | 250 ms | Reference |
| SS = 0.8 mV/s | 3 | (No peaks) | $-156.65 \pm (n=1)$ | (No peaks) | -26.98 ± 0.36 |
| | 20 | $-126.7 \pm 45.3^*$ | $-119.7 \pm 46.2^*$ | $-102.1 \pm 15.9^*$ | -34.17 ± 0.17 |
| | 200 | $-27.59 \pm 4.34^*$ | -23.91 ± 2.47 | $-23.43 \pm 3.64^*$ | -19.95 ± 0.27 |
| | 2000 | -30.82 ± 1.16 | -29.18 ± 0.42 | -29.39 ± 0.46 | -29.88 ± 0.23 |
| SS = 0.1 mV/s | 3 | $598.3 \pm (n=1)$ | $534.3 \pm 430.0^*$ | 1046 ± 301 | -26.98 ± 0.36 |
| | 20 | $763.3 \pm 216.2^*$ | -107.9 ± 105.0 | -45.69 ± 21.3 | -34.17 ± 0.17 |
| | 200 | -13.51 ± 17.2 | -19.51 ± 2.30 | -21.15 ± 4.01 | 19.95 ± 0.27 |
| | 2000 | -28.05 ± 1.88 | -64.91 ± 80.14 (-29.08 ± 1.27) ^b | -29.31 ± 0.40 | -29.88 ± 0.23 |
| Curve fit | 3 | -10.19 ± 54.03 | 0.93 ± 57.69 | -63.18 ± 29.03 | -26.98 ± 0.36 |
| | 20 | -53.43 ± 14.85 | -36.50 ± 14.72 | -38.50 ± 11.52 | -34.17 ± 0.17 |
| | 200 | $-21.65 \pm 0.56^*$ | -21.57 ± 1.18 | -21.05 ± 0.75 | -19.95 ± 0.27 |
| | 2000 | -30.82 ± 0.14 | -30.24 ± 0.59 | -30.40 ± 0.82 | -29.88 ± 0.23 |

* Statistically different from reference value ($p < 0.05$).

^b Mean and error for SS = 0.1 mV/s (2000 nA s) excluding one aberrant determination.

for signal levels below 200 nA s using this method. The SS = 0.1 method showed significant differences for only the 63 ms integration time, but gave very large errors for all but the 250 ms integration time. At the 3 nA s level, where the higher SS failed to detect peaks, the SS = 0.1 method produced large errors ($\delta^{13}\text{C} > 300$) and statistically different mean isotope ratios. In contrast, only one of twelve fitted means is different from the reference values (63 ms, 200 nA s) at 95% confidence.

The relatively high quality of the curve-fitted data at low levels prompted investigation of the preservation of peak area with decreasing sample size, for quantitative analysis. Graded solutions of pure Me17:0 in hexane were injected at levels from about 10 pmol to 500 nmol C. Areas for the m/z 44 signal were obtained by each of the calibration methods and summary data are plotted in Fig. 5, in log-log format. Peak areas below about 1 nmol are not accessible in our version of the vendor software and so are not presented. The SS = 0.8 method parallels the curve-fit method to 1 nmol, while the SS = 0.1 method diverges, with slopes and S.D.s for the

two methods of $6 \cdot 10^{-3} \pm 6 \cdot 10^{-6}$ ($r^2 > 0.999$) and $5 \cdot 10^{-3} \pm 20 \cdot 10^{-6}$ ($r^2 < 0.99$), respectively. The curve-fit method provides good linearity down to 10 pmol, with slope and S.D. of $5 \cdot 10^{-3} \pm 6 \cdot 10^{-6}$ ($r^2 > 0.999$) over this range.

The Finnigan MAT 252 instrument is specified to operate at an absolute sensitivity of 1000 CO_2 molecules/ion detected, and typically attains this

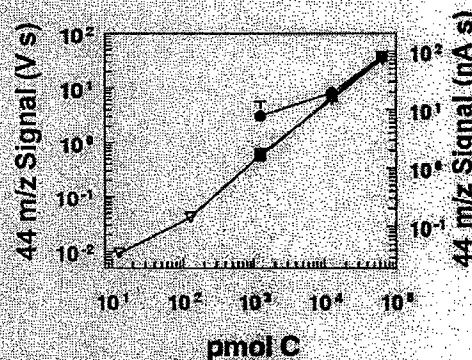


Fig. 5. Calibration curve showing the relationship of integrated area to mass of Me17:0 injected on-column for all three integration methods. ■ = SS 0.8 mV/s; ● = SS 0.1 mV/s; ▽ = curve fit.

level. For such an instrument, the 2000 nA s area would be produced with about 5 nmol C entering the combustion furnace, while the 3 nA s level would be attained with 5 pmol C. It should be emphasized that these detection limits are based on molar C content, and so are largely independent of the analyte molecular mass for most organic molecules of biomedical interest. These data indicate that curve fitting produces useful isotope ratios at this level of total analyte.

4. Conclusions

For tracer applications, a cutoff for "high precision" can be defined as an error of $\delta^{13}\text{C} = 100$ (S.D.), which corresponds to 0.1% in absolute terms and to the practical limit of organic mass spectrometers operated in selected ion mode (SIM) [2]. The $\text{SS} = 0.1$ mV/s detected peaks at levels below the $\text{SS} = 0.8$ mV/s method, but produced S.D.s greater than $\delta^{13}\text{C} = 100\text{‰}$ in most cases at the lower levels. $\text{SS} = 0.1$ did perform better for the largest integration time and lowest signal, further suggesting that the summation methods require a minimum S/N for adequate performance. For this reason, it appears that the higher value for SS is appropriate when using the summation method, as it detects peaks down to levels at which high precision is obtained. In cases where very small peaks must be detected by summation, consecutive data points can be summed to improve S/N per data point, with a lower SS then applied to the resulting chromatogram.

At all levels, curve-fitting produced satisfactory precision and accuracy, and was nearly immune to the choice of integration time. It was the only method that produced useful isotope ratios at the lowest levels. It also gives linear response over the entire usable dynamic range and so is also suitable for quantitative analysis. Our previous results showed that curve fitting satisfactorily recovers aberrant isotope ratios derived from overlapping peaks [12]. Together, these studies indicate that curve fitting may be the method of choice for integrating peaks in

high-precision chromatography applications where complex mixtures are commonly encountered.

Acknowledgements

This work was supported by NIH grant GM49209. K.J.G. acknowledges predoctoral support from NIH training grant DK07158.

References

- [1] D.E. Matthews and J.M. Hayes, *Anal. Chem.*, **50** (1978) 1465–1473.
- [2] K.J. Goodman and J.T. Brenna, *Anal. Chem.*, **64** (1992) 1088–1095.
- [3] R.J. Cairni and J.T. Brenna, *Anal. Chem.*, **65** (1993) 3497–3500.
- [4] R.J. Cairni and J.T. Brenna, *J. Mass Spectrom.*, (1995) in press.
- [5] D.A. Merritt and J.M. Hayes, *J. Am. Soc. Mass Spectrom.*, **5** (1994) 387–397.
- [6] T. Preston and C. Slater, *Proc. Nutr. Soc.*, **53** (1994) 363–372.
- [7] J.T. Brenna, *Acc. Chem. Res.*, (1994) in press.
- [8] D.A. Merritt and J.M. Hayes, *Anal. Chem.*, **66** (1994) 2336–2347.
- [9] S. Tissot, S. Normand, R. Guilluy, C. Pachiaudi, M. Beylot, M. Laville, R. Cohen, R. Mornex and J.P. Riou, *Diabetologia*, **33** (1990) 449–456.
- [10] Z.K. Guo, A.H. Luke, W.P. Lee and D. Schoeller, *Anal. Chem.*, **65** (1993) 1954–1959.
- [11] Y. Khalfallah, S. Normand, S. Tissot, C. Pachiaudi, M. Beylot and J.P. Riou, *Biol. Mass Spectrom.*, **22** (1993) 707–711.
- [12] K.J. Goodman and J.T. Brenna, *Anal. Chem.*, **66** (1994) 1294–1301.
- [13] A.H. Anderson, T.C. Gibb and A.B. Littlewood, *J. Chromatogr. Sci.*, **8** (1970) 640–646.
- [14] A.H. Anderson, T.C. Gibb and A.B. Littlewood, *Anal. Chem.*, **42** (1970) 434–440.
- [15] N. Dyson, *Chromatographic Integration Methods*, Royal Society of Chemistry, Cambridge, UK, 1990.
- [16] M.P. Ricci, D.A. Merritt, K.H. Freeman and J.M. Hayes, *Org. Geochem.*, **21** (1994) 561–571.
- [17] G.W. Snedecor and W.G. Cochran, *Statistical Methods*, Iowa State Univ. Press, Ames, IA, 1989.
- [18] K.J. Goodman and J.T. Brenna, presented at the 41st Annual ASMS Conference on Mass Spectrometry and Allied Topics, San Francisco, CA, 1993, abstracts, pp. 738a–738b.

Derivatization of Organic Compounds Prior to Gas Chromatographic-Combustion-Isotope Ratio Mass Spectrometric Analysis: Identification of Isotope Fractionation Processes

Gareth Rieley

School of Chemistry, University of Bristol, Bristol, UK BS8 1TS

An examination of the practice of derivatizing organic compounds such as fatty acids, sterols and amino acids in relation to subsequent analysis via gas chromatography-combustion-isotope ratio mass spectrometry is presented. Fractionation processes, such as kinetic isotope effects, which cause a deviation in the measured stable carbon isotope ratios (δ -values) of derivatized compounds from simple mass balance considerations are examined. Particular attention is paid to reactions that proceed by the cleavage of a carbon-containing bond and reactions that are likely to have kinetic isotope effects associated with them, such as acetylation and diazotization. Isotope fractionation processes other than those which are kinetic based are also discussed, as is the additional imprecision of the calculation of the δ -values of sample compounds inherent when derivative carbon is added. Failure to take this imprecision into account when comparing δ -values could lead to erroneous conclusions with respect to the magnitude of kinetic isotope effects caused by derivative reactions.

Keywords: Gas chromatography-combustion-isotope ratio mass spectrometry; derivatization; isotope ratio measurement; kinetic isotope effect; precision

Introduction

The ratios of the stable isotopes of elements important to living systems such as those of carbon, nitrogen, oxygen, hydrogen and sulfur ($^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$, $^{18}\text{O}/^{16}\text{O}$, $^2\text{H}/^1\text{H}$, and $^{34}\text{S}/^{32}\text{S}$, respectively), give information regarding many biological processes and transformations within global biogeochemical cycles.^{1,2} To measure these ratios (or δ -values), samples are generally combusted and the resultant gases cryogenically purified, the appropriate gas being subjected to isotope ratio mass spectrometry (IRMS). Recently, IRMS instruments have been connected to gas chromatographs via on-line combustion interfaces³⁻⁵ (this combined technique being called irmGC-MS, GC-IRMS or GC-C-IRMS), allowing for the measurement of stable isotopes of individual compounds from complex mixtures.⁶⁻⁸

This work concentrates on GC-C-IRMS analyses of carbon-containing compounds. As such, many compounds of interest will contain highly polar functional groups (e.g., carboxylic acid groups) and the problems associated with analysing such compounds by GC have been reviewed by Klee.⁹ It is possible to analyse these compounds directly using high-polarity stationary phases (e.g., FFAP-CB; Chrom-pack). However, these phases generally have maximum operating temperatures in the region of 250 °C, leading to long analysis times, broad peaks and reduced resolution, especially for compounds with carbon numbers in excess of C_{24} . The use

of low-polarity stationary phases (e.g., methylsilicone) for the analysis of polar compounds is also generally unsatisfactory owing to chromatographic adsorption effects such as peak tailing and loss of sample. The commonly adopted solution to such effects is to analyse polar compounds as less polar derivatives. Another commonly encountered problem in the examination of organic compounds is the analysis of the components of esters, whereby high relative molecular mass compounds are reduced to more amenable components through solvolysis reactions.²

Isotope Fractionation Processes

Only those processes that affect the measurement of the ratios of stable carbon isotopes are considered in the following discussion, although the principles are applicable to GC-C-IRMS analyses of other elements.

When the δ -value of a compound is required, and this compound has been derivatized before carbon GC-C-IRMS analysis, account must be taken of the contribution of derivative carbon to the measured δ -value of the derivatized compound. In principle, if there is no isotope fractionation process associated with the derivatization reaction, then the following mass balance equation will hold:

$$n_{cd}F_{cd} = n_cF_c + n_dF_d \quad (1)$$

where n is the number of moles of carbon, F is the fractional abundance of carbon-13, subscript c represents the compound of interest, d the derivative group and cd the derivatized compound.

F is defined in the following way:

$$F = \frac{[^{13}\text{C}]}{[^{12}\text{C} + ^{13}\text{C}]} \quad (2)$$

For natural abundance measurements, δ -values can be used to replace, F , the relationship between F and δ being as follows:²

$$F = \frac{R}{1 + R} \quad (3)$$

$$R = \frac{[^{13}\text{C}]}{[^{12}\text{C}]} \quad (4)$$

$$\delta = \left(\frac{R_{\text{standard}}}{R_{\text{sample}}} - 1 \right) \times 1000 \quad (5)$$

Hence, assuming the δ -values of the measured compounds are within natural abundance levels, eqn. (1) can be replaced with

$$n_{cd}\delta_{cd} = n_c\delta_c + n_d\delta_d \quad (6)$$

If compounds that are artificially enriched in ^{13}C are analysed, eqn. (1) must be used, and in the equations to follow δ -values should be replaced with fractional abundances.

The following sections discuss potential deviations from the mass balance eqns. (1) and (6).

Kinetic Isotope Effects

Kinetic isotope effects originate in the nature of vibrational energy levels of bonds; bonds involving heavier isotopes have a higher potential energy than those involving lighter isotopes,¹⁰ this effect being greater the stronger the bond. For example, a deuterium-carbon bond is stronger than a hydrogen-carbon bond. This difference can lead to differences in reaction rate for reactions involving different isotopic species, i.e., $k_2/k_3 \neq 1$, where k_2 is the reaction rate for the lighter isotope and k_3 that for the heavier isotope, the ratio of these giving the kinetic isotope effect. Usually, a kinetic isotope effect is greater than unity, owing to the heavier isotope bond being the stronger.

There are several possible kinetic isotope effects, the primary isotope effect being the most significant, whereby a bond containing the atom in consideration is changed (e.g., broken) in the rate-determining step.¹⁰ For deuterium, primary isotope effects are generally in the range 4–8.¹¹ For carbon-13 versus carbon-12, isotope effects are much smaller, owing to the relative mass of the carbon isotopes, usually being in the range 1.01–1.10.¹¹ These isotope effects are indicative of a change in bonding (usually bond cleavage) at a carbon position in the rate-determining step, if no carbon bond is changed in the rate-determining step, and indeed if no carbon-containing bond is involved in the reaction, then there is not likely to be a primary isotope effect.¹⁰

Another possible kinetic isotope effect is a secondary isotope effect. This involves the substitution of a heavier isotope at a position adjacent to the bond to be broken. However, this effect is unlikely to be of concern with regard to derivatization reactions, owing to the relatively small magnitude for carbon isotopes.¹⁰

If a kinetic isotope effect is to cause an isotope fractionation at a specific carbon position, there must be incomplete conversion of the reactant containing the carbon bond involved in the rate-determining step (non-quantitative reaction). This could be a reaction branch point, such as often occurs in biochemical reactions, or alternatively could be non-reaction or re-formation of reagents from a reaction intermediate. In derivatization reactions, the most likely effect will come from a non-quantitative reaction of one of the reagents, allowing for a kinetic isotope effect to be expressed at any carbon centre involved in the rate-determining step, and causing a fractionation of carbon isotopes at a specific position in the derivatized compound. If the reagent containing the carbon bond altered in the rate-determining step is in excess relative to other reagents, then full expression of the kinetic isotope effect would be expected, and in this event the maximum theoretical fractionation (as ‰) at a single carbon position is given by

$$\Delta = \left(\frac{k_{12}}{k_{13}} - 1 \right) \times 1000 \quad (7)$$

where Δ is the difference between the δ -value of the carbon position in the original compound and that in the derivatized compound. For example, a kinetic isotope effect of 1.02 would lead to a depletion in ^{13}C at a single carbon of up to 20‰.

If all the reagent were to react to form the derivative (quantitative reaction), then no fractionation would be expected ($\Delta = 0$).

In summary, for a significant kinetic isotope effect to occur at a carbon position causing an alteration in the δ -value at that position, the bond involving that carbon must be involved in the rate-determining step (i.e., affect k_2/k_3), and the reaction involving that bond must be non-quantitative.

Kinetic Isotope Effects With Regard to Derivatization

In many derivatization reactions, a hydrogen attached to a polar heteroatom (e.g., oxygen or nitrogen) is replaced with a group that leads to a lower polarity of the derivatized compound, and tends to increase the volatility of the compound.⁹ Both of these characteristics are desirable for the sharp resolution of compounds by normal-phase capillary columns (e.g., methylsilicone).⁹ Transesterification of esters is a slightly different case, whereby instead of a hydrogen atom, a hydrocarbon chain is replaced with the derivative group (usually a methyl group), giving an acyl ester and a free alcohol.

Silylation

For this work, derivatization reactions can be separated into two types, those that do not involve a carbon centre contained in the final derivative, e.g., silylation, and those that do, e.g., esterification reactions. As discussed earlier, those derivatization reactions which do not involve carbon-containing bonds will not lead to a kinetic isotope effect in the reaction. Hence silylation, which adds an alkylated silyl group to a hydroxy group, is not likely to have a kinetic isotope effect associated with the derivatization reaction which will affect the $\delta^{13}\text{C}$ value of a carbon position contained in the final derivative (for an example, see ref. 12).

Esterification

Esterification reactions, such as methylation, acetylation, isopropylation and transesterification proceed around a carbon centre contained in the final derivative, and thus a carbon kinetic isotope effect is possible. The mechanism of esterification reactions tends to proceed via the formation of a tetrahedral intermediate¹³ (e.g., Fig. 1), although other mechanisms are possible.¹³ An isotope fractionation effect centred on the carbonyl carbon is possible if the reaction is not forced to the formation of products with regard to that carbon centre, and the cleavage of a carbon-containing bond is involved in the rate-determining step.

In esterification and transesterification derivatization reactions that usually use an excess of an alcohol with a catalyst (e.g., ref. 14), the reactions are normally quantitative and rapid with regard to the carbonyl. Therefore, no kinetic isotope effect would be expected which alters the $\delta^{13}\text{C}$ value at that position, even if a kinetic isotope effect should be associated with the reaction. In general, this is an important point, in that no isotope fractionation effect will be expressed at a carbon position in the product if the reagent containing that carbon reacts quantitatively.

Acetylation

The esterification reactions discussed previously utilize an excess of an alcohol as a reagent. In contrast, acetylation

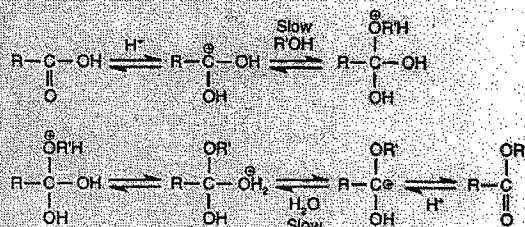


Fig. 1 A_{AC}^2 mechanism for esterification in acidic solution (after Ingold¹³)

derivatization reactions use an excess of an acid (usually as an acetate). Therefore, the carbonyl carbon involved in the rate-determining step of acetylation is not in the compound of interest, but rather in the derivative reagent. It follows that, as the acetate reagent is in excess, and will not react quantitatively, a kinetic isotope effect is possible at the acetyl carboxy-carbon, even though the compound of interest may react quantitatively.

Silfer *et al.*¹⁵ reported $\delta^{13}\text{C}$ analyses in which amino acids were converted into *N*-trifluoroacetyl (*N*-TFA) isopropyl esters. The procedure used was to isopropylate the amino acid carboxyl group using acidic propan-2-ol. The isopropyl amino esters were then acetylated with excess of trifluoroacetic anhydride with dichloromethane as solvent. Silfer *et al.* provided evidence that a simple mass-balance equation accounting for the addition of derivative carbon, such as that given by eqn. (6), is not adequate to account for the measured $\delta^{13}\text{C}$ value of the derivatized amino acid after the acetylation step (as discussed earlier, the isopropylation step should not alter the $\delta^{13}\text{C}$ value of the amino acid carbonyl). Silfer *et al.* suggested that a correction factor is required to account for this discrepancy, which is dependent on amino acid type and independent of the $\delta^{13}\text{C}$ value of the amino acid and of the anhydride used.

However, the results of Silfer *et al.* can be interpreted in a mechanistic manner. The 'correction factors' proposed can be rationalized as kinetic isotope effects. Thus, the rate-determining step of the trifluoroacetylation of an amino acid by trifluoroacetic anhydride in dichloromethane is the dissociation of the acetic anhydride (step 2 in Fig. 2), as opposed to protonation of the amine group¹⁶ (step 1 in Fig. 2). Examining the results of Silfer *et al.* and assuming that a kinetic isotope effect is centred only on the trifluoroacetyl carbon, a kinetic isotope effect can be calculated for this reaction of 1.019 (standard deviation = 0.003) (Table 1), presumably the variation of the individual isotope effects being due to substituent effects, different degrees of reaction or possible systematic errors. If this hypothesis is correct, then quantitative cleavage of the acetyl group from the derivatized amino acid and measurement of the $\delta^{13}\text{C}$ values of the acetate and the amino acid should show a difference with respect to ^{13}C abundance, relative to starting materials for the former, and not the latter, a more accurate measure of the kinetic isotope effect would also be gained by such an experiment. In addition, altering the acetylation reaction conditions so that protonation of the amine group becomes rate limiting (step 1) may obviate the need for correction factors. Use of alternative acetylation reagents, such as a trifluoroacetyl halide, and/or alternative reaction conditions, e.g., basic solution,¹⁶ may be preferable to those utilized by Silfer *et al.*¹⁵

Diazotization

Diazomethane is commonly utilized to methylate carboxyl, hydroxyl, thiol and amino groups;¹¹ a general reaction scheme is given in Fig. 3. In contrast to methylation reactions which use an excess of alcohol (e.g., BF_3 -methanol), diazotization involves a carbon centre contained in the final derivatized

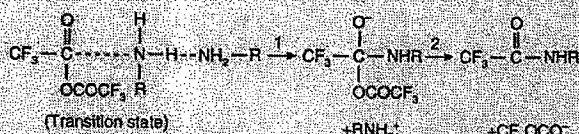


Fig. 2 Mechanism for the trifluoroacetylation of a primary amine. If step 1 is rate limiting, then no carbon isotope effect is expected; if step 2 is rate limiting, then a kinetic isotope effect is expected at the trifluoroacetyl carbonyl carbon. (Adapted from March¹¹)

compound which does not react quantitatively, but may be involved in the rate-limiting step of the reaction. If the rate-determining step is the dissociation of the diazo group from the methylated transition state (i.e., associated with the nucleophilic addition step, Fig. 3), then a kinetic isotope effect will be expressed, centred on the added methyl carbon, as the diazomethane reagent is in excess. Prevention of such an effect would be to choose reaction conditions so that protonation of the diazide is rate limiting, or simply not to methylate by such a route.

Intramolecular Differences in δ -Values Within Derivative Groups

Certain derivatization reagents have carbon-containing leaving groups, where the reagent 'splits,' with only part of the reagent molecule adding to the sample compound. Examples of this are silylating reagents, which add an alkylated silyl group to the sample compound and which can have an acetamide leaving group. In this case, the δ -value of the whole reagent will undoubtedly be different from the δ -value of the added silyl group, owing to the different sources of the latter and the acetamide group. In such instances, isotopic analysis of the bulk reagent would lead to an incorrect assignment of the average δ -value of the silyl carbons.

Consideration of Uncertainties Introduced When Utilizing Derivatives

The previous discussion has been largely concerned with isotope effects that could cause isotopic fractionation and thus

Table 1 ^{13}C kinetic isotope effects associated with the trifluoroacetylation of amino acid isopropyl esters using excess of trifluoroacetic anhydride in dichloromethane¹⁵

| Amino acid | n_c^a | n_{cd}^b | Correction, Δ^c | K.I.E. ^d |
|------------|---------|------------|------------------------|---------------------|
| Gly 1 | 2 | 7 | -2.37 | 1.0166 |
| Gly 2 | 2 | 7 | -2.34 | 1.0164 |
| D-Ala | 3 | 8 | -1.97 | 1.0158 |
| L-Ala | 3 | 8 | -2.30 | 1.0184 |
| D-Val | 5 | 10 | -2.46 | 1.0246 |
| L-Val | 5 | 10 | -2.17 | 1.0217 |
| D-Leu | 6 | 11 | -1.82 | 1.0200 |
| L-Leu | 6 | 11 | -1.81 | 1.0199 |
| D-Phe | 9 | 14 | -1.55 | 1.0218 |
| L-Phe | 9 | 14 | -1.65 | 1.0231 |
| D-Asp | 4 | 12 | -1.56 | 1.0187 |
| L-Asp | 4 | 12 | -1.65 | 1.0198 |
| D-Glu | 5 | 13 | -0.99 | 1.0129 |
| L-Glu | 5 | 13 | -1.11 | 1.0144 |
| D-Lys | 6 | 13 | -2.77 | 1.0180 |
| L-Lys | 6 | 13 | -2.95 | 1.0190 |

^a Number of carbons in the amino acid.

^b Number of carbons in the derivatized amino acid.

^c Correction factor, Δ , after Silfer *et al.*¹⁵ Δ is the difference between the measured and predicted $\delta^{13}\text{C}$ value of the derivatized amino acid.

^d Kinetic isotope effect, calculated from the equation $\text{K.I.E.} = -[1 + (\Delta n_{cd})/1000]$, where x is the number of amine groups in the amino acid, Δ is the correction factor and n_{cd} is the number of carbons in the derivatized amino acid.

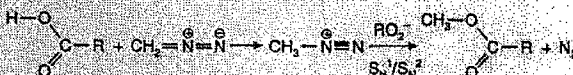


Fig. 3 General mechanism for the methylation of a carboxylic acid using diazomethane. (Adapted from March¹¹)

cause a deviation from an 'expected' δ -value predicted by simple mass balance. However, this section is concerned with the additional imprecision introduced into a corrected δ -value when derivative groups are added. If this imprecision is large, and ignored, then incorrect assumptions as to the magnitude of isotope effects are possible. Thus, for example, if the imprecision in a calculated value is $\pm 0.6\%$, and the difference between the expected δ -value and that measured by a different technique (e.g., comparison of GC-C-IRMS and conventional IRMS techniques) is 0.5% , then conclusions that the difference is due to an isotope effect may be incorrect.

Theoretical Considerations

Most derivatization reactions add one derivative group to one functional group in the sample molecule (other occurrences are dealt with in the Appendix). For the simplest case, the δ -value of the derivatized compound can be calculated using a mass-balance equation such as given earlier in eqn. (6). As the unknown δ -value is that of the compound of interest, δ_c , the δ -value of the derivatized compound, and that of the derivative group being known, eqn. (6) can be rearranged to give

$$\delta_c = \frac{n_{cd}\delta_{cd} - n_d\delta_d}{n_c} \quad (8)$$

The value calculated with this equation is the δ -value that would be obtained if there were no errors associated with the analytical measurements of δ_{cd} and δ_d , i.e., no imprecision. However, this is unlikely to be the case, and for routine measurement

$$\delta_c = \delta_{c, \text{meas}} \pm \epsilon_c \quad (9)$$

where ϵ_c is the uncertainty in the instrument measurement of δ_c . For GC-C-IRMS this is currently about $\pm 0.3\%$. Techniques that obtain the $\delta^{13}\text{C}$ value of whole samples (i.e., conventional IRMS), as opposed to individual compounds, can attain a precision from $\pm 0.05\%$ to better than $\pm 0.03\%$,^{17,18} although for individual compounds this precision should be tempered with possible systematic errors due to sample impurities.

In order to determine the uncertainty associated with the calculation of the δ -value of a compound of interest, ϵ_c , the uncertainty in the isotope value of the derivative group, ϵ_d , and the uncertainty in the isotope value of the derivatized compound, ϵ_{cd} , need to be taken into account. The general equation for the combination of errors, where a measurement X is a function of the variables A, B, \dots , etc., each having errors associated with them given by $\pm \epsilon_A, \pm \epsilon_B, \dots$, etc., is given by

$$\epsilon_c^2 = \left(\frac{\partial X}{\partial A}\right)^2 \epsilon_A^2 + \left(\frac{\partial X}{\partial B}\right)^2 \epsilon_B^2 + \dots \quad (10)$$

assuming no covariance of those variables.¹⁹ Solving eqn. (10) for eqn. (8) gives

$$\epsilon_c^2 = \epsilon_d^2 \left(\frac{n_d}{n_c}\right)^2 + \epsilon_{cd}^2 \left(\frac{n_c + n_d}{n_c}\right)^2 \quad (11)$$

If the δ -value of the derivative group is measured indirectly, for example by measuring the δ -value of a standard, δ_s , and that of the derivatized standard (e.g., for silylation¹²), δ_{sd} , then additional uncertainty is introduced. The δ -value of the derivative group can be calculated using a rearranged eqn. (8), and the associated error of δ_c is given by

$$\epsilon_c^2 = \epsilon_s^2 \left(\frac{n_s}{n_c}\right)^2 + \epsilon_{sd}^2 \left(\frac{n_s + n_d}{n_c}\right)^2 + \epsilon_{cd}^2 \left(\frac{n_c + n_d}{n_c}\right)^2 \quad (12)$$

Note: even though the isotopic compositions of the standard and the derivatized standard, δ_s , and δ_{sd} , covary, the

instrumental measurements do not, and the term for covarying errors $[2(\partial X/\partial A)(\partial X/\partial B)\sigma_{A,B}]$ will equal zero and hence is left out of eqns. (10) and (12).¹⁹

Discussion of Imprecision

If the imprecision associated with the utilization of derivative groups is to be minimized, then the smaller the relative contribution to the final derivatized compound from derivative carbon, the smaller is the associated error. This mainly becomes a problem with compounds with less than ten carbons (e.g., amino acids, monosaccharides and phenolics; see Fig. 4), where derivative carbon becomes a major factor. In these situations, minimization of the error associated with assignment of the δ -value of the derivative carbon becomes critical, especially if this assignment is indirect (e.g., ref. 12).

If indirect assignment of the δ -value of the added derivative carbon is used, then minimization of the associated error is possible maximizing the contribution of derivative carbon relative to that of the standard used. Hence a low carbon number standard with multiple derivatizable groups will help to minimize the error. It should be noted that failure to take into account increases in uncertainty may lead to incorrect conclusions as to isotope effects.

Conclusions

If derivatives are to be used when analysing organic compounds by GC-C-IRMS, then the possibility of a kinetic isotope effect should be considered. Close attention to the mechanisms of derivative reactions will allow for sites of potential kinetic isotope effects to be highlighted, and for reaction conditions to be tailored so that such isotope effects are not expressed. The use of internal standards that have the same functional group to be derivatized in a sample compound, and of which the δ -value is known, is an advisable way

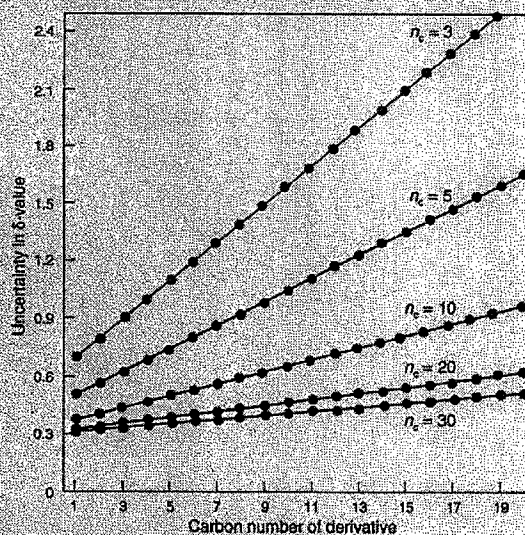


Fig. 4. Uncertainty in the calculation of a compound's δ -value versus the carbon number of a derivative added to the compound, for several different compound carbon numbers, using eqn. (11). Only one derivative group is added to both the standard and the compound. n_c is the carbon chain length of the compound of interest. The isotope standard is assumed to contain 16 carbons. The instrumental errors in the measurement of the δ -values of the isotope standard, the derivatized standard and the derivatized compound are 0.03, 0.1 and 0.3, respectively. (All errors are %).

in which to check for possible isotope effects. It should also be noted that the additional imprecision of δ -values of sample compounds, added when using derivative groups, must be considered before conclusions as to isotope effects are drawn.

The author thanks S. Lam, J. F. Carter, J. W. Collister, G. S. S. Pearce and J. M. Hayes for discussions during manuscript preparation.

Appendix

The following mathematical treatment indicates general equations for the use of derivative groups prior to GC-C-IRMS analyses. The key to the symbols used is as follows:

Operators:

n_x = the number of carbon atoms in compound x ;
 δ_x = the delta value of x ;
 ϵ_x = the uncertainty in the value of δ_x ;
 a_i, b_i = the number of derivative groups of type i added to a compound;
 z = the number of different derivative groups of type a_i or b_i added to a compound

Subscripts

c = a compound of interest;
 s = any isotope standard;
 d = a derivative group;
 i = the different types of derivative group added to a compound, e.g., δ_d is the calculated δ -value of a derivative group.

In the most general case, when a compound containing z different functional groups, of which there are a_i of each functional group contained in the compound, is derivatized with z different derivatives, then the theoretical resultant δ -value is given by

$$\delta_{cd} = \frac{n_c \delta_c + \sum_{i=1}^z a_i n_{d_i} \delta_{d_i}}{n_c + \sum_{i=1}^z a_i n_{d_i}} \quad (\text{A1})$$

To calculate the δ -value of the derivatized compound of interest, eqn. (8) can be rearranged to give

$$\delta_c = \frac{\left(n_c + \sum_{i=1}^z a_i n_{d_i}\right) \delta_{cd} - \sum_{i=1}^z a_i n_{d_i} \delta_{d_i}}{n_c} \quad (\text{A2})$$

The associated error of δ_c is given by

$$\epsilon_c^2 = \sum_{i=1}^z \frac{\epsilon_{d_i}^2 n_{d_i}^2}{n_c^2} + \epsilon_{cd}^2 \left(\frac{n_c + n_d}{n_c} \right)^2 \quad (\text{A3})$$

If indirect means are used to determine the δ -value of the derivative groups, where isotope standards are used, of which the δ -value is known, then the δ -value, δ_d , will be given by

$$\delta_d = \frac{n_{cd} \delta_{cd} - n_c \delta_c}{b n_d} \quad (\text{A4})$$

where b is the number of functional groups derivatized in the isotope standard. If several derivatives are used that are calibrated in this way, then the error in the calculation of δ_c will then be given by

$$\epsilon_c^2 = \sum_{i=1}^z \frac{\epsilon_{d_i}^2 n_{d_i}^2}{n_c^2} + \sum_{i=1}^z \frac{\epsilon_{cd}^2 (n_c + b_i n_{d_i})^2}{n_c^2} + \epsilon_{cd}^2 \left(\frac{n_c + \sum_{i=1}^z a_i n_{d_i}}{n_c} \right)^2 \quad (\text{A5})$$

References

- Griffiths, H., *Funct. Ecol.*, 1991, 5, 254.
- Hayes, J. M., in *Organic Geochemistry of Contemporaneous and Ancient Sediments*, Society of Economic Paleontologists and Mineralogists, Bloomington, IN, 1983, ch. 5.
- Matthews, D. E., and Hayes, J. M., *Anal. Chem.*, 1978, 50, 1465.
- Hayes, J. M., Freeman, K. H., Popp, B. N., and Hoham, C. H., *Org. Geochem.*, 1989, 16, 1115.
- Bakin, P. A., Fallick, A. E., and Gere, J., *Chem. Geol.*, 1992, 101, 71.
- Freeman, K. H., Hayes, J. M., Trendel, J.-M., and Albrecht, P., *Nature (London)*, 1989, 343, 254.
- Rieley, G., Collier, R. J., Jones, D. M., Eglinton, G., Eakin, P. A., and Fallick, A. E., *Nature (London)*, 1991, 352, 425.
- Collister, J. W., Summons, R. E., Lichfouse, E., and Hayes, J. M., *Org. Geochem.*, 1993, 19, 265.
- Klec, M. S., in *Modern Practice of Gas Chromatography*, ed. Grob, R. L., Wiley, New York, 2nd edn., 1985.
- Melander, L., and Saunders, W. H., *Reaction Rates of Isotopic Molecules*, Wiley, New York, 1980.
- March, J., *Advanced Organic Chemistry: Reactions, Mechanisms and Structure*, Wiley, New York, 3rd edn., 1985.
- Jones, D. M., Carter, J. F., Eglinton, G., Jumeau, E. J., and Fenwick, C. S., *Biol. Mass Spectrom.*, 1991, 20, 641.
- Ingold, C. K., *Structure and Mechanism in Organic Chemistry*, Cornell University Press, Ithaca, NY, 2nd edn., 1969.
- Goodman, K. J., and Brenna, J. T., *Anal. Chem.*, 1992, 64, 1088.
- Silfer, J. A., Engel, M. H., Macko, S. A., and Jumeau, E. J., *Anal. Chem.*, 1991, 63, 370.
- Blackburn, G. M., and Jencks, W. P., *J. Am. Chem. Soc.*, 1968, 90, 2638.
- Handley, L. L., Scrimgeour, C. M., Thornton, S. F., and Sprent, I. I., *Funct. Ecol.*, 1991, 5, 119.
- Fry, B., Brand, W., Mersch, P. J., and Garritt, R., *Anal. Chem.*, 1992, 64, 288.
- Bevington, P. R., *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York, 1969.

Paper 3/04405J

Received July 26, 1993

Accepted October 14, 1993

Reprinted from

JOURNAL of CHROMATOGRAPHY

Journal of Chromatography A, 752 (1996) 233–241

Influence of gas chromatographic parameters on measurement of $^{13}\text{C}/^{12}\text{C}$ isotope ratios by gas–liquid chromatography–combustion isotope ratio mass spectrometry. I

W. Meier-Augenstein^{a,*}, P.W. Watt^a, C.-D. Langhans^b

^a*University of Dundee, Dept. of Anatomy and Physiology, Small's Wynd, Dundee DD1 4HN, UK*

^b*University Children's Clinic, Dept. of Neuropaediatrics, Stable Isotope Laboratory, Im Neuenheimer Feld 130, D-69120 Heidelberg, Germany*

Received 10 April 1996; revised 13 May 1996; accepted 23 May 1996



Influence of gas chromatographic parameters on measurement of $^{13}\text{C}/^{12}\text{C}$ isotope ratios by gas–liquid chromatography–combustion isotope ratio mass spectrometry. I

W. Meier-Augenstein^{a,*}, P.W. Watt^a, C.-D. Langhans^b

^aUniversity of Dundee, Dept. of Anatomy and Physiology, Small's Wynd, Dundee DD1 4HN, UK

^bUniversity Children's Clinic, Dept. of Neuropaediatrics, Stable Isotope Laboratory, Im Neuenheimer Feld 150, D-69120 Heidelberg, Germany

Received 10 April 1996; revised 13 May 1996; accepted 23 May 1996

Abstract

A potential influence of gas chromatographic parameters on measured $^{13}\text{C}/^{12}\text{C}$ -isotope ratios of saturated fatty acid methyl esters in gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) has been studied. A dependence of measured isotope ratios on temperature gradients was observed and differences in measured $\delta^{13}\text{C}$ -values for individual fatty acid methyl esters varied by 0.5 to 3‰. Possible reasons for this isotopic fractionation and its implications for ^{13}C natural abundance work are discussed.

Keywords: Combustion isotope ratio mass spectrometry; Isotope effects; Fatty acid methyl esters; Carbon 13

1. Introduction

Since the commercial availability of gas isotope ratio mass spectrometers (IRMS) directly coupled to a gas chromatograph (GC) via an on-line combustion interface (C), based on the design by Matthews and Hayes [1], GC–C–IRMS has become a powerful tool in all areas of applied analytical chemistry [2].

One inherent problem of this technique, however, has attracted little interest so far. The analysis of medium to high boiling point organic compounds by gas–liquid chromatography (GLC) is associated with two potential sources for isotopic fractionation. Kinetic isotope effects are associated with all de-

rivatisation reactions that proceed by the cleavage or the formation of a carbon-containing bond. This effect has been noted for example in trifluoroacetylation of amino acids [3] and an in-depth examination of this effect in general has been reported recently [4].

Apart from that kinetic isotope effect there also exists a thermodynamic isotope effect that is associated with GLC of isotopically substituted compounds [5]. This effect is commonly known as the "inverse" chromatographic isotope effect [6] because the heavier isotopomer elutes earlier than the unlabelled substance. For ^{14}C -labelled fatty acids of higher molecular weight this effect can be observed by gas chromatography mass spectrometry [7]. To the best of our knowledge, its consequences for GC–C–IRMS measurements of organic compounds of natu-

*Corresponding author.

ral abundance in ^{13}C have not been investigated so far.

In this paper, we report on isotopic fractionation of saturated fatty acid methyl esters, naturally abundant in ^{13}C , observed during $\delta^{13}\text{C}$ measurements at varying gas chromatographic conditions. A tentative explanation for the surprisingly high magnitude of the isotope effect and conclusions as to practical consequences are presented.

2. Experimental

2.1. Chemicals

Individual fatty acid methyl esters (FAME) and National Health Institute (NHI) FAME reference mixtures were obtained from Sigma (St. Louis, MO, USA). Solvents were obtained from Fluka (Buchs, Switzerland) and were of >99.7% purity.

Sample 1 was prepared by mixing 2 μl of each individual FAME (C6, C10, C11, C12 and C14) and diluting this mixture in 1 ml of *n*-heptane. Sample 2 was prepared by adding 2 μl of each C6 and C11 FAME to 10 μl of FAME mixture NHI-C, containing FAMES C8 through to C20 in increasing weight%, and diluting the resulting mixture in 1 ml of *n*-heptane.

2.2. Gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS)

Studies were carried out on an ORCHID GC–C–IRMS system (Europa Scientific, Crewe, UK) unless stated otherwise. The combustion interface, comprising a combustion furnace operated at 820°C (CuO/Pt wires), a Nafion tube acting as water separator, and a reduction furnace operated at 600°C (Cu wires) transformed sample peaks eluted from the GC into dry CO_2 and N_2 . In ^{13}C -studies, measured isotope ratios are automatically corrected for contributions from ^{17}O [8] and expressed as δ -values in per mille [‰] units:

$$\delta^{13}\text{C} = (R_{\text{sample}} - R_{\text{standard}}) / R_{\text{standard}} \times 1000 \quad (1)$$

where R_{sample} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the sample and R_{standard} is the $^{13}\text{C}/^{12}\text{C}$ ratio of the working standard. Since this study was only concerned with changes

rather than absolute values, in all experiments methylcaproate (C6) was initially chosen as internal standard and its $\delta^{13}\text{C}$ -value was arbitrarily set to zero.

2.3. Gas chromatography (GC)

The gas chromatograph was an HP 5890, Series II, fitted with an electronic pressure control (EPC) thus permitting the carrier gas management to be set either to constant pressure or to constant flow mode.

Helium of 5.0 purity was used as carrier gas and was passed through a high capacity gas purifier (Supelco, Poole, UK) before entering the GC. Column head pressure was controlled by an EPC. In either mode, carrier gas was set to one of three linear-velocities at a column temperature of 70°C. These velocities were 22.1 cm/s, 28.4 cm/s and 35.4 cm/s, respectively.

Separation of saturated FAMES was achieved on columns coated with stationary phases of different polarity: a CP-Sil 8 CB capillary column (Chrompack, Middelburg, Netherlands), dimensions 25 m \times 0.25 mm, 0.25 μm film thickness and a CP-Sil 19 CB, dimensions 25 m \times 0.25 mm, 1.2 μm film thickness. The columns were connected to a retention gap, 1 m \times 0.25 mm, that was cyanopropyl/phenyl/methyl deactivated [9].

Column temperature was held at 70°C for 6 min and subsequently programmed to 270°C at one of three temperature gradients. These gradients were 4°C/min, 6°C/min and 8°C/min, respectively. Under these conditions methylcaproate (C6) always eluted at the end of the isothermal step ($t_R = 387$ s) on the CP-Sil 8 CB column and thus was not subject to any change in GC conditions. For this reason methylcaproate (C6) was initially referred to as internal standard.

The injector temperature was set to 230°C, split flow to 30 ml/min, and identical aliquots of samples 1 and 2 were injected in split and splitless mode, respectively. In case of the latter, split was kept closed for initial 6 s and kept open afterwards. Sample volumes injected were 0.5 μl and 0.2 μl for split and splitless mode, respectively. Thus, in splitless mode 1.74 nmol of methylundecanoate (C11) was injected on the column. With a split ratio of 1:40 at the open split in front of the mass spectrometer ion

source 522 pmol of CO_2 entered the ion source giving rise to a m/z 44 signal area of 12.9 nA s.

2.4. Data analysis

Data were analysed using the manufacturer provided software (Europa Scientific ORCHID POST-PROCESSOR). Peaks are detected by comparing calculated ascending and descending gradients with user-defined threshold slopes for peak start and stop. In this case, slope thresholds of +3 pA/s and -3 pA/s were chosen for start and stop, respectively. Once peak start and stop have been defined for channel 1, i.e. beam m/z 44 in the case of CO_2 , the corresponding beams simultaneously measured on channel 2 and 3 are automatically associated with channel 1 at any point in time. Peaks are automatically baseline corrected.

Once a chromatographic peak has been identified, the isotope ratio for that compound is determined by a linear regression fit. The software plots e.g. beam 1 (x axis) against beam 2 (y axis) to produce a profile of the ratio as we move from the bottom of the peak to the top of the peak. A least squared fit (LR^2) is applied to the scatter plot, the result of which is a slope of the line which corresponds to the isotope ratio. The LR^2 fit has the shape of an ellipse which is a function of the isotope shift. The 2/1 and 3/1 ratios of the analytes and the reference compound are determined in exactly the same way.

Data collected on the Delta S (Finnigan, Bremen,

Germany) was analysed using the Finnigan software ISODAT. Peaks are detected by calculating start and stop slope for one data channel (beam 1= m/z 44) and comparing the result to user-defined threshold slopes. Slope thresholds were set to 0.35 mV/s. Peaks are automatically baseline corrected. Once the peak start and stop have been defined for the single channel, the values are extrapolated to the other two channels. Peak maxima are determined for all channels and a time shift is used to correct for chromatographic separation of isotopes [10–13].

3. Results and discussion

3.1. Influence of temperature gradient

Setting the carrier gas to a linear velocity of 28.4 cm/s at the initial temperature of 70°C, sample 1 was analysed at three different temperature gradients and two different carrier gas modes for each gradient. Furthermore, in order to investigate any potential influence of the design of the open split in front of the mass spectrometer on the outcome of the measurement, this experiment was repeated substituting an open split according to Hayes [14] for the open split-cum-isolation valve design used in the ORCHID.

The first experiments with sample 1 clearly showed the presence of an isotope effect (Table 1).

Table 1
Dependence of relative $\delta^{13}\text{C}$ -values of split injected saturated fatty acid methyl esters on temperature gradient

| | C10 Const. pressure ^a | C11 Const. pressure ^a | C12 Const. pressure ^a | C14 Const. pressure ^a |
|----------------------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| <i>Orchid open split</i> | | | | |
| 4°C/min | -8.41±0.04 | -8.09±0.15 | -6.32±0.18 | -6.89±0.06 |
| 6°C/min | -6.09±0.11 | -5.84±0.15 | -3.76±0.17 | -5.22±0.12 |
| 8°C/min | -5.10±0.11 | -4.88±0.09 | -3.36±0.21 | -4.73±0.18 |
| $\Delta\delta$ (4°–8°) | +3.31±0.12 | +3.21±0.17 | +2.96±0.28 | +2.16±0.19 |
| <i>J. Hayes open split</i> | | | | |
| 4°C/min | -8.00±0.08 | -7.87±0.13 | -5.63±0.19 | -6.54±0.26 |
| 6°C/min | -6.12±0.03 | -5.49±0.16 | -3.85±0.20 | -5.21±0.14 |
| 8°C/min | -5.50±0.21 | -4.73±0.11 | -3.37±0.09 | -4.47±0.10 |
| $\Delta\delta$ (4°–8°) | +2.50±0.22 | +3.14±0.22 | +2.26±0.21 | +1.83±0.28 |

All relative $\delta^{13}\text{C}$ -values in [‰] are given as mean ± standard deviation of 5 repetitions. Identical aliquots of sample 1 were injected in split mode. Split flow was set to 30 ml/min.

^a Column head pressure of 14.4 p.s.i. corresponding to a flow-rate of 1.12 ml/min and a linear velocity of 28.4 cm/s at a column temperature of 70°C. Column used was a CP-Sil 8 CB.

The shift towards higher ^{13}C -enrichment was independent of the design of the open split in front of the mass spectrometer ion source.

To rule out the influence of isotopic fractionation during injection (see below) and to study the extent of this effect on higher boiling FAMES further experiments with sample 2 were carried out using splitless injection.

The results of these experiments are presented in Fig. 1A. Data were evaluated and tested for significant difference on the basis of 95% confidence limits as well as the observed precision ($\pm 2\sigma$). Choosing C6 as internal reference peak, a significant shift from lower to higher δ -values could be detected when the results obtained for 4°C/min were compared with those obtained for 6°C/min and 8°C/min. On changing from 4°C/min to 8°C/min ramp rate, the difference in δ -values ranged from +2.58 to +1.02‰ with differences between 4 and 8°C/min being 2 to 3 times higher than corresponding differences between 6 and 8°C/min. The $\delta^{13}\text{C}$ -values for FAMES C14 to C20 measured at 6 and 8°C/min showed no significant differences within 2σ .

The way samples were injected showed, not surprisingly, a noticeable effect. The δ -values for FAME C11 should have shown no variation for a given set of GC parameters when compared to results obtained for sample 1, yet they were shifted to higher δ -values by 1.43‰ on average when samples were injected splitless for 6 s. In the absence of any other variables this effect was attributed to isotopic fractionation in the injector of the relatively low boiling FAME C6 that was used as internal standard.

In order to rule out influences beyond the control of a standardised protocol, we re-evaluated our data from the experiments with sample 2 setting either C11 or C18 as the active internal reference peak (Fig. 1B and C). With C11 as reference peak, $\delta^{13}\text{C}$ -values for FAMES C14 to C20 were no longer significantly different within 2σ . When C18 was chosen as reference peak all but two $\delta^{13}\text{C}$ -values became virtually identical ($\pm 2\sigma$).

These results prompted us to repeat these experiments on a column coated with a stationary phase of medium polarity. Polymethylsiloxane (PMS) phases that have a 7% phenyl, 7% cyanopropyl substitution (e.g. CP-Sil 19 CB) generally show a higher selec-

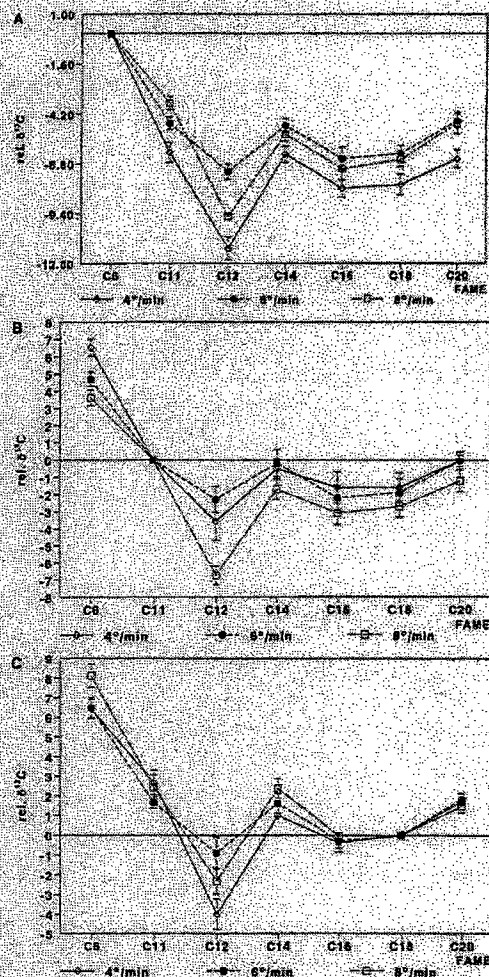


Fig. 1. Effect of GC parameters and choice of reference peak on relative $\delta^{13}\text{C}$ -values without time shift correction. Identical aliquots of sample 2 were injected splitless (split closed for 6 s) on a CP-Sil 8 CB column. Linear carrier gas velocity was set to 28.4 cm/s at a column temperature of 70°C. Values are the mean of 5 repetitions. Error bars are $\pm 2\sigma$. (A) $\delta^{13}\text{C}$ -values calculated vs. $\delta^{13}\text{C}(\text{C6})=0.000\text{‰}$. (B) $\delta^{13}\text{C}$ -values calculated vs. $\delta^{13}\text{C}(\text{C11})=0.000\text{‰}$. (C) $\delta^{13}\text{C}$ -values calculated vs. $\delta^{13}\text{C}(\text{C18})=0.000\text{‰}$.

tivity for FAMES than apolar phases such as CP-Sil 5 CB (100% PMS) or PMS with a 5% phenyl substitution (e.g. CP-Sil 8 CB).

Interestingly enough, with this stationary phase $\delta^{13}\text{C}$ -values for all FAMES were identical within $\pm 2\sigma$

when calculated using C6 as internal reference peak (Fig. 2A). When calculated vs. C11 as internal reference peak $\delta^{13}\text{C}$ -values for C16, C18 and C20 obtained at 4°C/min were significantly higher by 2‰ on average compared to their corresponding $\delta^{13}\text{C}$ -values obtained at 6 and 8°C/min (Fig. 2B). When C18 was set as internal reference peak all but three $\delta^{13}\text{C}$ -values became virtually identical within $\pm 2\sigma$.

Since all experiments were carried out under standardised conditions with respect to sample, injected sample volume, carrier gas velocity, temperature gradients, data evaluation and instrumental set-up, the different outcome of the experiments depicted in Figs. 1 and 2 could only be attributed to the differences in stationary phase properties. Furthermore, these findings clearly demonstrate that the calculated isotopic abundance depends on the choice of reference peak, i.e. on the overall chromatographic conditions (GC plus combustion interface) at the point in time represented by this peak. Hence, when no time shift correction is employed to compensate for chromatographic isotope effects the choice of internal reference peak has a significant impact on observed $\delta^{13}\text{C}$ -values.

The same set of experiments with sample 2 was carried through on another GC-C-IRMS instrument (Delta S). Since Finnigan's software ISODAT employed a time shift correction to compensate for chromatographic separation of isotopomers [10–13] we anticipated not being able to observe this effect here. This, indeed, was the case as only a slight trend towards lower ^{13}C -values was detected when changing the temperature gradient from 8°C/min to 4°C/min (Fig. 3). However, the measured changes in $\delta^{13}\text{C}$ -values ranging from 0.13 to 0.65‰ lay well within the observed precision ($\pm 2\sigma$).

When we compared the results from both instruments obtained at 6°C/min on an apolar stationary phase with C6 as internal reference peak, time shift corrected $\delta^{13}\text{C}$ -values were on average 1.31‰ higher.

3.2. Influence of carrier gas flow

The studies on the influence of temperature gradient on $^{13}\text{C}/^{12}\text{C}$ isotope ratios were carried out by setting the carrier gas flow to its optimum linear velocity of 28.4 cm/s [15] corresponding to a flow-

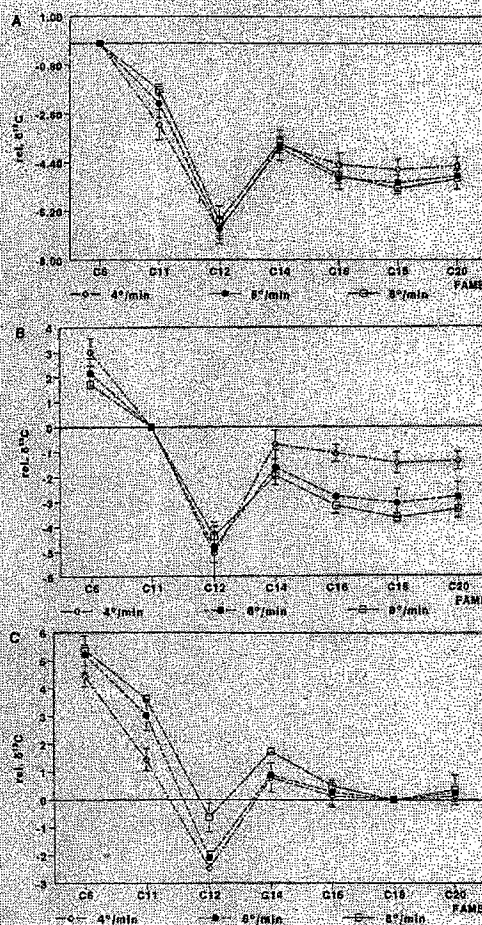


Fig. 2. Effect of GC parameters and choice of reference peak on relative $\delta^{13}\text{C}$ -values without time shift correction. Identical aliquots of sample 2 were injected splitless (split closed for 6 s) on a CP-Sil 19 CB column. Linear carrier gas velocity was set to 28.4 cm/s at a column temperature of 70°C. Values are the mean of 3 repetitions. Error bars are $\pm 2\sigma$. (A) $\delta^{13}\text{C}$ -values calculated vs. $\delta^{13}\text{C}(\text{C6})=0.000\text{‰}$; (B) $\delta^{13}\text{C}$ -values calculated vs. $\delta^{13}\text{C}(\text{C11})=0.000\text{‰}$; (C) $\delta^{13}\text{C}$ -values calculated vs. $\delta^{13}\text{C}(\text{C18})=0.000\text{‰}$.

rate of 1.12 ml/min. To monitor a potential influence of actual carrier gas flow, these studies were run under both constant pressure and constant flow conditions. When a constant column head pressure is applied the actual carrier gas flow decreases as the temperature increases because of the gas viscosity-temperature dependency. To compensate for that, in

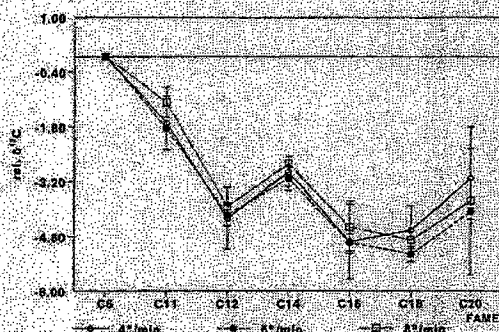


Fig. 3. Effect of GC parameters and choice of reference peak on relative $\delta^{13}\text{C}$ -values when time shift correction is employed (Delta S). Identical aliquots of sample 2 were injected splitless (split closed for 6 s) on a CP-Sil 5 CB column. Linear carrier gas velocity was set to 36 cm/s at a column temperature of 70°C. Values are the mean of 3 repetitions; error bars are $\pm 2\sigma$; $\delta^{13}\text{C}$ -values were calculated vs. $\delta^{13}\text{C}(\text{C6}) = 0.000\%$.

constant flow mode the column head pressure is increased electronically over the temperature gradient.

Although the observed differences in $\delta^{13}\text{C}$ -values were not significantly different, at temperature gradients of 6 and 8°C/min a trend towards slightly lower $\delta^{13}\text{C}$ -values going from constant pressure to constant flow mode could be noticed and the results for 6°C/min are presented in Table 2.

Analyses run at higher column head pressure or higher flow rate yielded slightly lower $\delta^{13}\text{C}$ -values as compared with $\delta^{13}\text{C}$ -values observed at lower pressure or lower flow rate (Table 2), an effect that appeared to decrease with increasing chain length. Comparing results between a constant pressure setting and its corresponding constant flow setting also did not show significant differences. Once again a trend could be detected showing a slight ^{13}C -depletion for FAMES run at constant flow compared with $\delta^{13}\text{C}$ -values obtained from the FAMES when run at corresponding constant pressure mode.

3.3. Discussion

Although the observation of an isotope effect in GC-combustion came as no surprise, however, the magnitude of this effect was unexpected. The influence of GC parameters on the separation of stable

isotope labelled compounds from their unlabelled analogues is well known [6,16–19]. Employing e.g. ^2H -labelled compounds, this effect is exploited in stable isotope dilution analysis as well as for internal standardisation and quantification [20] because introduction of deuterium atoms into a molecule results in a significantly decreased retention time [21]. Matucha et al. [7] investigated this phenomenon on deuterated *n*-alkanes and ^{14}C -labelled methyl esters of palmitic and oleic acid. Uniformly ^{14}C -labelled FAMES eluted 1.8 s earlier than their unlabelled analogues. Matucha and coworkers clearly showed that this phenomenon was caused by lower molar volumes of the labelled, and thus heavier, compounds and resulting changes in chromatographic solute-stationary phase interaction. Our own observations showed that for di-tBDMS derivatives of $^2\text{H}_3$ -labelled L-leucine and unlabelled L-leucine separation increased by 0.9 s going from 8°C/min to 4°C/min at a constant pressure of 8 p.s.i. and increased by 0.48 s going from 8 p.s.i. to 16 p.s.i. at a temperature gradient of 6°C/min.

As to the exact nature of the isotope effect observed in this study we can but speculate. However, our observations are consistent with the assumption that the separation of isotopomers in the GC column, i.e. solute-stationary phase interaction dominated by Van der Waals dispersion forces leading to an earlier elution of the heavier isotopomer [7], is one key factor in the observed isotope effect.

To test this hypothesis we analysed sample 2 again using a temperature gradient of 4°C/min but having set the column head pressure to 18.2 p.s.i. Under these conditions the heavier isotopomers should elute even earlier and, hence, should result in even lower $\delta^{13}\text{C}$ -values compared with the values obtained for 4°C/min at 14.4 p.s.i. This experiment yielded $\delta^{13}\text{C}$ -values on average 1‰ lower than those observed for 4°C/min and 14.4 p.s.i. which substantiated our findings.

Using different temperature gradient under constant column head pressure had a markedly effect on retention time and the separation of the FAMES. Peak shapes however, were not significantly effected (Table 3). Despite the considerable changes in separation of the individual FAMES we were not able to determine the difference in retention times for the isotopomers. Time difference between mass

Table 2
Dependence of relative $\delta^{13}\text{C}$ -values of splitless injected saturated fatty acid methyl esters on carrier gas parameters

| | 11.1 p.s.i. Const. pressure ^a | 0.8 ml/min Const. flow ^b | 14.4 p.s.i. Const. pressure ^a | 1.12 ml/min Const. flow ^b | 18.2 p.s.i. Const. pressure ^a | 1.54 ml/min Const. flow ^b | $\Delta\delta$ (11.1-18.2 p.s.i.) | $\Delta\delta$ (0.8-1.54 ml/min) |
|----------------------------|---|--|---|---|---|---|--------------------------------------|-------------------------------------|
| <i>J. Hayes open split</i> | | | | | | | | |
| C10 | -1.37±0.89 | -1.64±1.05 | -2.11±0.81 | -3.34±0.66 | -2.23±1.00 | n.d. | -0.86±1.30 | n.d. |
| C11 | -4.35±0.19 | -4.89±0.05 | -5.05±0.04 | -5.34±0.03 | -5.12±0.31 | -5.56±0.12 | -0.77±0.36 | -0.67±0.13 |
| C12 | -3.48±0.22 | -3.57±0.17 | -3.72±0.52 | -3.67±0.07 | -0.386±0.67 | -3.38±0.09 | -0.38±0.71 | +0.19±0.10 |
| C13 | -3.92±0.18 | -3.86±0.16 | -3.97±0.22 | -4.32±0.08 | -3.91±0.11 | -4.39±0.03 | +0.01±0.21 | -0.53±0.16 |
| C16 | -6.59±0.13 | -6.69±0.13 | -6.97±0.43 | -6.76±0.08 | -6.65±0.18 | -6.89±0.20 | -0.06±0.23 | -0.20±0.24 |
| C18 | -6.36±0.10 | -6.80±0.14 | -6.79±0.31 | -6.85±0.12 | -6.67±0.20 | -6.90±0.11 | -0.31±0.22 | -0.10±0.18 |
| C20 | -4.76±0.22 | -5.08±0.15 | -5.01±0.25 | -5.18±0.02 | -5.01±0.13 | -5.05±0.10 | -0.25±0.26 | +0.03±0.18 |

All relative $\delta^{13}\text{C}$ -values in [‰] are given as mean±standard deviation of 5 repetitions. Identical aliquots of sample 2 were injected in splitless mode. Split was kept closed for 6 s and opened thereafter for the rest of the run. Split flow was set to 30 ml/min. Column temperature was held at 70°C for 6 min and then programmed to 270°C at 6°C/min. Column used was a CP-Sil 8 CB.

^a Column head pressure of 11.1 p.s.i. corresponding to a flow-rate of 0.80 ml/min and a linear velocity of 22.1 cm/s at a column temperature of 70°C.

^b Column head pressure of 14.4 p.s.i. corresponding to a flow-rate of 1.12 ml/min and a linear velocity of 28.4 cm/s at a column temperature of 70°C.

^c Column head pressure of 18.2 p.s.i. corresponding to a flow-rate of 1.54 ml/min and a linear velocity of 35.4 cm/s at a column temperature of 70°C.

Table 3
Retention times t_R and peak width t_w of saturated fatty acid methyl esters for various temperature gradients

| | C6 | C11 | C12 | C14 | C16 | C18 | C20 |
|----------------------|------------------|----------|----------|----------|----------|----------|----------|
| 4°C/min | t_R 387±0.5 | 1613±0.5 | 1812±0.5 | 2177±0.5 | 2507±0.5 | 2807±0.5 | 3084±0.5 |
| | t_w 30±0.5 | 32±0.5 | 24±0.5 | 27±0.5 | 28±0.5 | 28±0.5 | 29±0.5 |
| 6°C/min | t_R 387±0.5 | 1329±0.5 | 1466±0.5 | 1714±0.5 | 1938±0.5 | 2142±0.5 | 2329±0.5 |
| | t_w 30±0.5 | 31±0.5 | 24±0.5 | 27±0.5 | 28±0.5 | 28±0.5 | 29±0.5 |
| 8°C/min | t_R 387±0.5 | 1168±0.5 | 1274±0.5 | 1463±0.5 | 1634±0.5 | 1789±0.5 | 1934±0.5 |
| | t_w 29±0.5 | 30±0.5 | 22±0.5 | 24±0.5 | 25±0.5 | 25±0.5 | 27±0.5 |
| Δt_R (8-4°C) | 0 | 445 | 538 | 714 | 873 | 1018 | 1150 |
| Δt_w (8-4°C) | 1 | 445 | 538 | 3 | 3 | 3 | 2 |

Retention times t_R and peak width t_w (s) are given as mean±standard deviation of 5 repetitions. Identical aliquots of sample 2 were injected in splitless mode. Split was kept closed for 6 s and opened thereafter for the rest of the run. Split flow was set to 30 ml/min.

^a Column head pressure of 14.4 p.s.i. corresponding to a flow-rate of 1.12 ml/min and a linear velocity of 28.4 cm/s at a column temperature of 70°C. Column used was a CP-Sil 8 CB.

traces m/z 44 and m/z 45 was of the order of 100 ms apex to apex, but, changes from one temperature gradient to another were too small to be determined with sufficient precision.

However, the surprisingly high magnitude of this isotope effect for samples of natural abundance in ^{13}C in conjunction with one internal reference peak could be due to an amplification of this effect caused by the combustion interface. Since the compounds eluting from the column are combusted into CO_2 (and H_2O), a molecule of low molecular mass and as such much more susceptible to isotopic fractionation, that is subsequently transferred into the mass spectrometer passing on its way numerous connections and changes in capillary diameter, it can be speculated that the passage through the combustion interface amplifies the isotope effect in GLC. The distortion of peak shapes by the combustion interface and its resulting influence on precision has been discussed by Goodman and Brenna [13].

4. Conclusion

The isotope effect in GC-C-IRMS and the various influences of GC parameters on this effect have been confirmed for compounds of natural abundance in ^{13}C . It is small but reliably measurable with modern isotope ratio mass spectrometers thus adding studies relating to the theory of gas chromatography to the list of GC-C-IRMS applications, provided the data evaluation software allows to calculate the isotope ratios without time shift correction. On the other hand, the results of this study show that time shift correction is one powerful tool to compensate for any isotope effect occurring during GC-combustion.

Especially in typical GC-C-IRMS applications of natural abundance work such as food adulteration, aroma and perfume adulteration, forensics and environmental studies, the isotope effects occurring in GLC should be taken into account.

Practical consequences are (1) once established, to stick to one protocol for a certain application, (2) to inject samples splitless to avoid potential isotopic fractionation of lower boiling compounds, (3) not to compare, or only with caution, $\delta^{13}\text{C}$ -values for a given compound obtained by different methods and

different instruments, and (4) rather than comparing "absolute" values, to compare $\delta^{13}\text{C}$ -values from an unknown sample with those from an authentic sample measured under identical conditions and/or to apply the "finger print" method [22].

Furthermore, these findings emphasise the necessity to use an internal standard of calibrated isotope ratio that is subject to the same physical conditions as the analytes of interest introduced into the GC-C-IRMS system [2]. The results reported in this paper show that adding one internal standard to the sample to be analysed may not be enough since the magnitude of the isotope effect varies over the course of the GC analysis and depends on the relative position of the reference peak in the chromatogram. Adding an internal standard mixture, however, will create unwanted problems caused by co-elution or at least overlap of analytes and individual internal standards. Investigations as to how this problem can be overcome are almost finished and will be reported at a later date [23,24].

Acknowledgments

This work was supported by the European Community, grant no. MATI-CT-94031 as part of the third framework.

References

- [1] D.E. Matthews and J.M. Hayes, *Anal. Chem.*, **50** (1978) 1465–1473.
- [2] J.F. Brenna, *Acc. Chem. Res.*, **27** (1994) 340–346 and references cited therein.
- [3] J.A. Silfer, M.H. Engel, S.A. Macko and E.J. Jumeau, *Anal. Chem.*, **63** (1991) 370–374.
- [4] G. Rieley, *Analyst*, **119** (1994) 915–919.
- [5] P. Krumbiegel, *Isotopieeffekte*, Akademie-Verlag, Berlin, Germany (1970) p. 135.
- [6] Y. Cherrah, J.B. Falconnet, M. Desage, J.L. Brazier, R. Zini and J.P. Tillement, *Biomed. Environ. Mass Spectrom.*, **14** (1987) 653–657.
- [7] M. Matucha, W. Jokisch, P. Verner and G. Anders, *J. Chromatogr.*, **588** (1991) 251–258.
- [8] H. Craig, *Geochim. Cosmochim. Acta*, **12** (1957) 133–149.
- [9] G.R. van der Hoff, P. van Zoonen and K. Grob, *J. High Resolut. Chromatogr.*, **17** (1994) 37–42.

- [10] M. Rautenschlein, K. Habfast and W.A. Brand, in: *Stable Isotopes in Paediatric, Nutritional and Metabolic Research*, Intercept, Andover, 1990 pp 133-148.
- [11] K.H. Freeman, J.M. Hayes, J.M. Trendel and P. Albrecht, *Nature*, 343 (1990) 254-256.
- [12] K.J. Goodman and J.T. Brenna, *Anal. Chem.*, 66 (1994) 1294-1301.
- [13] W.A. Brand, *J. Mass Spectrom.*, 31 (1996) 225-235.
- [14] D.A. Merrit, W.A. Brand and J.M. Hayes, *Org. Geochem.*, 21 (1994) 573-583.
- [15] K. Grob, *Making and Manipulating Capillary Column for Gas Chromatography*, Huethig, Heidelberg, 1986 p. 193.
- [16] T. Hanai, *J. High Resolut. Chromatogr.*, 13 (1990) 178-181.
- [17] M. Matucha, *Chromatographia*, 27 (1985) 552-558.
- [18] Y.A. Zolotarev, D.A. Zarsev, M.Y. Lubnin, V.Y. Tatur and N.F. Myasoyedov, *Appl. Radiat. Isot.*, 39 (1988) 619.
- [19] M. Mohinke and J. Heybey, *J. Chromatogr.*, 471 (1989) 37-53.
- [20] W. Meier-Augenstein, G.F. Hoffmann, B. Holmes, J.L. Jones, W.L. Nyhan and L. Sweetman, *J. Chromatogr.*, 615 (1993) 127-135.
- [21] T. Goromaru and H. Maeda, *Biol. Pharm. Bull.*, 17 (1994) 1635-1639.
- [22] R. Braunsdorf, U. Hener, S. Stein and A. Mosandl, *Z. Lebensm. Unters. Forsch.*, 197 (1993) 137-141.
- [23] Patent pending.
- [24] W. Meier-Augenstein, *Anal. Chem.* (1996) submitted.



Isotope-ratio-monitoring gas chromatography-mass spectrometry: methods for isotopic calibration

DAWN A. MERRITT,^{1,*} W. A. BRAND² and J. M. HAYES¹

¹Biogeochemical Laboratories, Departments of Chemistry and of Geological Sciences, Geology Building, Indiana University, Bloomington, IN 47405, U.S.A. and ²Finnigan MAT, Barkhausenstr. 2, 2800 Bremen, Germany

Abstract—In trial analyses of a series of *n*-alkanes, precise determinations of ¹³C contents were based on isotopic standards introduced by five different techniques and results were compared. Specifically, organic compound standards were coinjected with the analytes and carried through chromatography and combustion with them; or CO₂ was supplied from a conventional inlet and mixed with the analyte in the ion source; or CO₂ was supplied from an auxiliary mixing volume and transmitted to the source without interruption of the analyte stream. Additionally, two techniques were investigated in which the analyte stream was diverted and CO₂ standards were placed on a near-zero background. All methods provided accurate results. Where applicable, methods not involving interruption of the analyte stream provided the highest performance ($\sigma = 0.00006$ at. % ¹³C or 0.06‰ for 250 pmol C as CO₂ reaching the ion source), but great care was required. Techniques involving diversion of the analyte stream were immune to interference from coeluting sample components and still provided high precision ($0.0001 \leq \sigma \leq 0.0002$ at. % or $0.1 \leq \sigma \leq 0.2$ ‰).

Key words—carbon-13, isotope ratio, mass spectrometry, analysis

INTRODUCTION

Isotopic analysis can be viewed as incorporating two steps: (i) measurement of ion-current ratios and (ii) conversion of the ion-current ratios into isotopic abundances. For high-precision measurements, the latter step involves "calibration", or expression of isotopic abundances *relative to* those in some primary standard. For conventional, batchwise analyses, this is facilitated by the design of the measurement. Sample and "standard" gases are alternately admitted to a mass spectrometer, the ion-current ratios deriving from them are compared, and relative isotopic abundances calculated (e.g. Santrock *et al.*, 1985). The analyst can control the delivery of gases to the ion source and the procedure can be readily optimized. That element of control is sacrificed when isotope-ratio-monitoring techniques (Merritt and Hayes, 1994) are applied to gas chromatographic effluents. For many natural samples (e.g. extracts of sediments or of biological specimens, crude oils), the continuous elution of compounds complicates isotopic calibration. On the one hand, conditions and times for introduction of standards should match those of analytes as closely as possible. On the other, introduction of standards should not interfere with

observation of analytes. The tension generated by these conflicting objectives can be reduced if the instrument is highly stable and frequent calibration therefore not required. That issue has been systematically explored in separate investigations and it has been shown that, for uncertainties in ¹³C/¹²C of about one part in 10⁴, calibration at 20-min intervals can be adequate (Merritt and Hayes, 1994).

But questions remain concerning optimal procedures for introduction of standards and these are investigated here. A variety of approaches is available: individual compounds can be coinjected, chromatographically separated, and combusted along with analytes (Hayes *et al.*, 1990); CO₂ can be introduced to the ion source from the variable-volume inlet or by injecting CO₂ into the carrier gas stream (Barrie *et al.*, 1984); or CO₂ can be admitted via a second helium stream (Freedman *et al.*, 1988). Here, we evaluate and compare these methods. Additionally, we consider the advantages of diversion of the GC effluent stream so that standards appear against minimal and consistent backgrounds. Two methods for accomplishing this diversion are compared and evaluated.

EXPERIMENTAL SECTION

Instrumentation

As shown in Fig. 1, the isotope-ratio-monitoring gas chromatography-mass spectrometry (irmGCMS)

*Present address: Environmental Chemistry Laboratory, DowElanco, Box 68955, Indianapolis, IN 46268-1053, U.S.A.

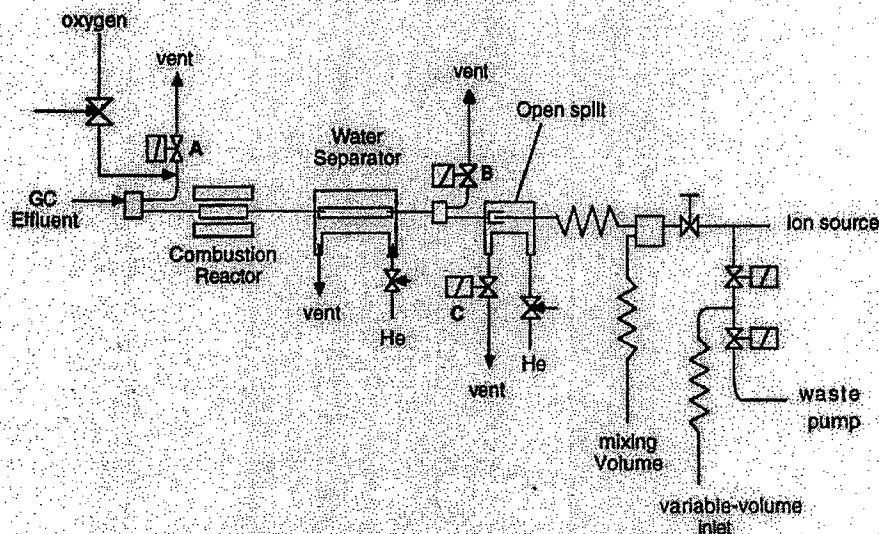


Fig. 1. Schematic diagram of combustion interface and related inlets.

system consisted of a gas chromatograph interfaced to an isotope-ratio mass spectrometer via a combustion and water-removal assembly. Individual components are described below.

Combustion interface. The system utilized either a Series 5890A (Hewlett-Packard, Avondale, PA) or a Model 3400 (Varian, Sugarland, Tex.) gas chromatograph. The exit of the chromatographic column was connected to a union with a two-hole ferrule on the downstream side (Scientific Glass Engineering, Austin, Tex.). Outputs were connected to a vent line controlled by an air-actuated "T" valve (valve A, SGE) and a combustion reactor operated at 850°C (CuO) or 1100°C (NiO). Water generated from the combustion of organic material was removed as the effluent stream passed through a 0.6-mm-i.d. tubular nafion membrane. The capillary (0.32-mm-i.d.) exiting the tubular membrane entered a second union with a two-hole ferrule on the downstream side. One output (0.32-mm i.d., 5-cm length) was inserted into the open split described below, while the other served as a vent line which was controlled by an air-actuated valve (valve B). The capillary carrying the effluent was centered within the open split, and a constant stream of dry helium (2 ml/min) passed through the housing at all times, exiting through a vent line controlled by an air-actuated valve (valve C).

The rate of admission of gas to the mass spectrometer was controlled by the conductance of a 0.11-mm-i.d. capillary inserted approx. 1 cm into the effluent line within the open split. At its low-pressure end, this capillary entered an isolation valve through a 1/4- to 1/16-in. Swagelok reducing union fitted with a graphitized-velspen two-hole ferrule; a second capillary leak which delivered a gas stream of

helium or 0.1–0.4% CO₂ in helium (2-m-length, 0.060-mm-i.d. deactivated vitreous silica, throughput: 0.02–0.04 ml/min) also entered the ion source through this union. The length of the capillary leak which delivered the effluent stream was determined experimentally such that the pressure in the ion source was approx. 4–6 μ Torr and the volumetric flow rate of carrier gas entering the mass spectrometer was 0.2–0.3 ml/min (10-kV instrument, capillary length = 1.2–2.0 m) or 0.5–0.8 ml/min (3-kV instrument, capillary length = 70–100 cm).

The gas stream entering the ion source through the second capillary leak was prepared using a device which controlled the relative positions of three capillaries inside a mixing volume: a 0.11-mm-i.d. capillary which delivered CO₂ (<0.03 ml/min of 100% CO₂ or 1 ml/min of 0.7% CO₂ in He), a 0.32-mm-i.d. capillary which delivered helium (4 ml/min), and the 0.06-mm-i.d. capillary leak which delivered a gas stream (0.02–0.04 ml/min) to the ion source [Fig. 2(b)]. The positions of the helium capillary and the capillary leak were fixed whereas the CO₂ capillary was mounted on a sliding arm attached to a piston whose position was controlled by valve E. The sliding arm moved this capillary over a maximum range of 4–5 cm and had two positions: 0.5–1 cm above the capillary leak (valve open) or 1–2 mm from the bottom of the mixing chamber (valve closed). In normal operation, the CO₂ capillary was positioned above the capillary leak so that only helium entered the ion source. To introduce CO₂, valve E was closed so that the CO₂ capillary dropped to the bottom of the chamber, mixing CO₂ into the gas below the entrance of the capillary leak. The relative flow rates of the CO₂ and helium streams entering the mixing chamber were adjusted so that the gas stream sent to

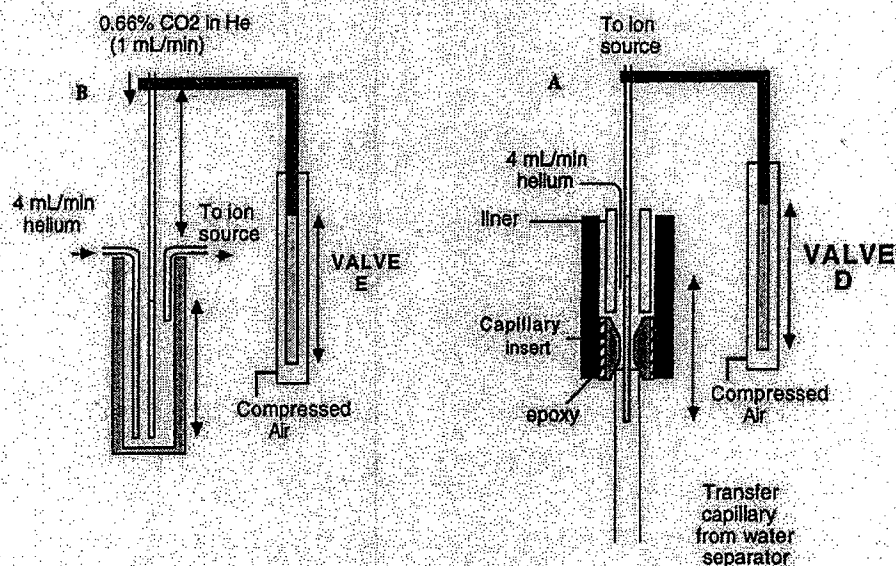


Fig. 2. (a) Schematic diagram of mixing chamber for intermittent preparation of CO_2/He standard gas. The capillary marked "to ion source" is connected to the inlet of the isolation valve shown in Fig. 1. (b) Schematic diagram of the diversion device described in the text. As indicated, the capillary carrying the combustion effluent to this device can be connected directly to the outlet of the water-removal system. In the present experiments, it was connected to the capillary leaving the union connected to valve B, thus taking the place of the open split.

the ion source contained 0.1–0.4% CO_2 in helium (v/v).

Mass spectrometers. Instruments with accelerating potentials of 3 kV (Delta S; Finnigan MAT, Bremen, Germany) or 10 kV (MAT 252; Finnigan MAT) were used in these experiments. Ions were generated by electron impact (70 eV), and the source and analyzer regions were separately pumped. The 10-kV instrument is considerably more efficient (1 CO_2 molecular ion per 900 molecules vs 1 CO_2 molecular ion per 30,000 molecules for the 3-kV instrument) and thus requires smaller samples for any given level of performance. Ion currents were measured continuously for m/z 44, 45, and 46 using triple Faraday cups connected to high-speed amplifiers. Small, constant currents were injected at the summing points of the electrometers. The resulting voltage offsets, which provided background signal ratios equal to those of CO_2 with natural isotopic abundances, were 90–110, 110–130, and 140–160 for m/z 44, 45, and 46, respectively.

Flow pathways

Four operational modes were available: normal/sampling, effluent-stream diversion, backflush, and oxidation. Pathways of gas streams were selected by computer-controlled, air-actuated valves. The system has been described in detail elsewhere (Merritt *et al.*, 1994). Here, we note that the backflush mode was always used to exclude solvent peaks from the interface and mass spectrometer and briefly describe

the normal/sampling and effluent-stream-diversion modes.

Normal/sampling. In this mode, valves A and B were closed, vent C was open to the atmosphere, and an auxiliary stream of helium (2–5 mL/min) flowed through the open split. As a result, the entire effluent stream passed through the combustion reactor and a portion of the combustion products entered the ion source. In most cases, the effluent flow rate was greater than that required by the mass spectrometer. Through operation of the open split, therefore, only a portion entered the ion source (0.2 or 0.5 mL/min, respectively, for the 10 and 3-kV instruments) while the remainder exited through vent C. If the flow rate of the effluent stream was less than that required by the mass spectrometer, the deficit was supplied by the auxiliary helium stream that constantly purged the open split. In typical operation, the volumetric flow rate of the effluent stream was 1 mL/min at a GC column temperature of 60°C. Therefore, 20–30% of the effluent stream entered the ion source of the 10-kV instrument (50–85% for the 3-kV instrument). Because the flow rate of carrier gas was controlled only by regulation of the column head pressure and thus declined as column temperature increased, the fraction of the effluent stream entering the ion source increased over the course of a temperature-programmed run.

Effluent-stream diversion. At any time during an analysis, the effluent stream could be diverted to the atmosphere by opening valve B while simultaneously

closing valve C. An alternative means of effluent diversion was also examined. In this case, the device shown in Fig. 2(b) took the place of the open split. As shown, an air-actuated piston provided for linear movement of the MS-inlet capillary, either inserting it a distance of 1 cm within the bore of a capillary carrying the full effluent stream or retracting it to a position 2.5–3 cm from the exit of that capillary. In the latter case, the MS-inlet capillary sampled only effluent-free He supplied by an auxiliary stream. In an optimized system, this unit would take the place of both the open split and the union connected to valve B. For the experiments performed here, the latter union was left in place and connected to the transfer capillary shown in Fig. 2(b).

Experimental procedures

Samples and sample introduction. A mixture containing a homologous series of *n*-alkanes (*n*-C₁₀–*n*-C₁₄; Aldrich, Milwaukee, Wis. and Sigma Chemical, St Louis, Mo.) was prepared. Solutions of this mixture contained approx. 20 nmol C/ μ l (3-kV instrument) or 5 nmol C/ μ l (10-kV instrument) per component. These mixtures were injected onto an open tubular, fused-silica column via an on-column injector (Hewlett-Packard) Ultra-1 (Hewlett-Packard: 50 m \times 0.32 mm \times 0.52 μ m) and RT-1 (Restek, Bellefonte, Pa.: 60 m \times 0.32 mm \times 0.5 μ m) columns were used routinely. Injected sample sizes were approx. 2 and 10 nmol C per component for the 10 and 3-kV instruments, respectively, with 0.4–0.6 and 5–8.5 nmol C actually entering the mass spectrometers. The chromatographic effluent was diverted from the combustion interface by closing valve C and opening valve A during the first 10 min of each analysis (Merritt *et al.*, 1994). For comparison, the isotopic compositions of all test compounds were measured by off-line combustion followed by conventional isotope-ratio mass spectrometry (Frazer and Crawford, 1963; Santrock *et al.*, 1985).

Introduction of isotopic standards. Two methods were used to introduce isotopic standards. In the first, a mixture containing five perdeuterated *n*-alkanes was coinjected with sample mixtures (5 or 25 nmol C/ μ l of C₁₆D₃₄, C₂₀D₄₂, C₂₄D₅₀, C₃₂D₆₆ and C₃₈H₇₈; MSD Isotopes, St Louis, Mo.). In the second method, CO₂ was introduced directly into the ion source through capillaries with limiting conductances. This was accomplished by introducing pure CO₂ from the variable-volume inlet or by using the mixing chamber described above to add CO₂ to the He stream entering the ion source through the second capillary leak (Fig. 1). In either case, the CO₂ standard could either be superposed on the GC-combustion effluent or the effluent stream could be diverted (by use of valves B and C or by use of the air-actuated diverter). The isotopic compositions of all standards were established by separate analyses using conventional techniques.

Calculations and presentation of results

Notation. Isotope ratios are expressed in terms of δ and reported in units of parts per thousand, or permil (‰).

$$\delta^{13}\text{C}_{\text{PDB}} = 10^3(^{13}\text{R} - ^{13}\text{R}_{\text{PDB}})/^{13}\text{R}_{\text{PDB}}$$

where $^{13}\text{R} \equiv ^{13}\text{C}/^{12}\text{C}$ and $^{13}\text{R}_{\text{PDB}}$ is the isotope ratio for the Pee Dee Belemnite primary standard. Because only carbon isotopic compositions are considered in this report and the PDB standard has been used in all cases, the notation is here simplified by use of δ in place of $\delta^{13}\text{C}_{\text{PDB}}$. Procedures for the calculation of δ developed by Santrock *et al.* (1985) and adapted for use in isotope-ratio-monitoring measurements by Ricci *et al.* (1994) were used in this work. Results are also reported in terms of Δ values in order to express the accuracy of isotopic measurements:

$$\Delta \equiv \delta_m - \delta_e$$

where the subscripts m and e refer to measured and expected (or "true") values of δ . To summarize the quality (combining both accuracy and precision) of a set of *n* measurements of δ values, we report "rms Δ ", root-mean-square Δ :

$$\text{rms } \Delta = (\sum \Delta^2/n)^{1/2}$$

RESULTS AND DISCUSSION

Standards introduced during normal operation

Coinjected internal standards. To calibrate isotopic measurements for individual compounds in a sample, the most purely differential procedure—with the standard sharing not only mass spectrometric conditions but also conditions of chromatography and combustion with the sample—is one in which compounds with known δ values are coinjected with the sample onto the chromatographic column. In principle, any compound which did not coelute with analytes, which was thermally and chemically stable in solution and in the chromatographic column, and which could be isolated in a pure form would be suitable. In practice, perdeuterated *n*-alkanes were chosen because they shared chemical and chromatographic properties but not retention times with the homologous *n*-alkanes used as test samples.

Ion-current (*m/z* 44) and ratio traces (45/44) are shown in Fig. 3 for an irmGCMS analysis of the test mixture of *n*-alkanes. Values of Δ are plotted in Fig. 4 for each compound in the series, the perdeuterated *n*-alkanes having been used as coinjected isotopic standards. The average and rms Δ s were 0.013 and 0.12‰, respectively. If arbitrarily selected *n*-alkanes (C₁₇, C₂₂, C₂₈, C₃₄) were used as isotopic standards (this being possible because the accurate δ values for these test materials were known), the average and rms Δ values were equivalent: 0.001 and 0.12‰, respectively. These results demonstrate the absence of systematic errors (average Δ not significantly different from zero) and contribute to the first

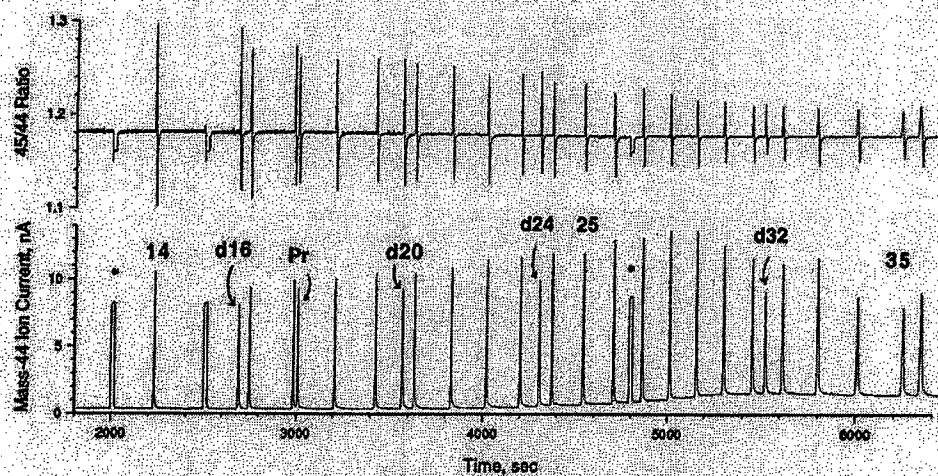


Fig. 3. Ion-current and ratio traces produced using the 10-kV instrument. The lower trace displays the m/z 44 ion current and the upper trace displays the instantaneous 45/44 ion-current ratio. Each peak represents 200–300 pmol CO_2 (RT-1 column, $60 \leq T \leq 320^\circ\text{C}$ at $3^\circ\text{C}/\text{min}$, 20 min hold at 320°C). Asterisks designate CO_2 -gas standards, numbers (and unmarked peaks) indicate homologous n -alkanes; d16 (etc.) designates the C_{16} deuteriocarbon standard; Pr, pristane.

entry in Table 1, which summarizes extensive experience with the a variety of calibration procedures. The entry for combustion of "coinjectured compounds" establishes a level of quality to which the performance of alternative procedures can be compared.

Admission of CO_2 from variable-volume inlet. Together with its associated capillary leak, the variable-

volume inlet is designed to produce a precisely controlled flow of CO_2 ideally suited for conventional, differential measurements. As indicated by the roughly square "peaks" superposed on the ion-current chromatogram in Fig. 3, it can also be used to deliver standard CO_2 in isotope-ratio-monitoring runs. The corresponding deflections in the ratio trace

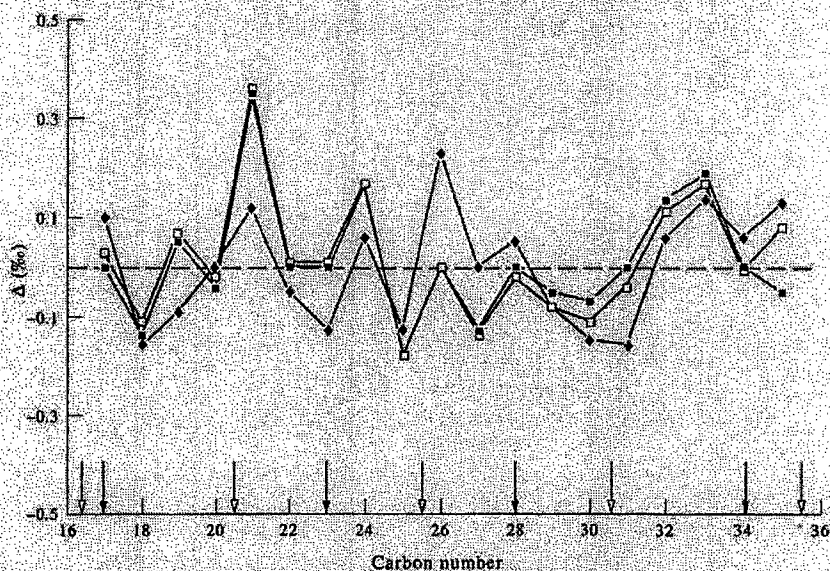


Fig. 4. Values of Δ for homologous n -alkanes in a single chromatogram in which δ values were based on three different methods of calibration: (i, solid diamonds) coinjected internal standards (perdeuterated n -alkanes) (C_{16} , C_{20} , C_{24} , C_{32} , and C_{36}), (ii, solid squares) selected n -alkanes indicated by solid arrows on the x -axis, and (iii, open squares) CO_2 delivered from the variable-volume inlet at the times marked by open arrows. These data were generated using the 3-kV instrument. Each peak represented 3–5 nmol CO_2 . Chromatographic conditions as for Fig. 3.

Table 1. Performance of calibration methods

| Type of isotopic standard | Diversion of effluent | avg. Δ (%) | rms Δ (%) |
|---|-----------------------|-------------------|------------------|
| Cojected compounds | No | $< \pm 0.03$ | 0.08-0.15 |
| CO ₂ , variable-volume inlet | No | $< \pm 0.05$ | 0.06-0.15 |
| CO ₂ , via second leak | No | $< \pm 0.05$ | 0.06-0.15 |
| CO ₂ , individual background | Yes | $< \pm 0.05$ | 0.10-0.15 |
| CO ₂ , single background | Yes | $< \pm 0.05$ | 0.12-0.20 |

indicate that the ion-current ratio (and thus the isotopic composition) was constant within each peak from the variable-volume inlet, in marked contrast to the enrichment-depletion cycle associated with each chromatographically separated peak (for discussion, see Ricci *et al.*, 1994). Several potential advantages are associated with this method of standardization. Because the ion-current ratio is constant within each peak, subdivision will not introduce any systematic errors. Editing techniques (Ricci *et al.*, 1994) can be used to redefine "peak-start" and "peak-stop" times in order to eliminate interferences that may affect a portion of the standard peak. Moreover, the square peak profile delivers, on average, more CO₂ per unit of peak width than a gaussian peak with equal height and width. Consequently, the precision of related isotopic analyses is improved. Finally and most obviously, it is attractive to have a single material (for which the isotopic composition can be established by a single conventional analysis) that can be applied as a standard at any point within a chromatogram.

As shown in Fig. 3, both CO₂ and perdeuterated standards were incorporated in a single chromatographic run. As for the perdeuterated standards, Δ values associated with analyses based on the CO₂ standards are plotted in Fig. 4. The average and rms Δ values were 0.016 and 0.12%, respectively, nearly identical to those obtained using the cojected perdeuterated standards. This observation documents an estimate based on hundreds of less well controlled observations made in the course of applications of irmGCMS and entered in Table 1, namely that neither of these techniques is inherently superior to the other *when the system is operating precisely as intended*. This last point will be discussed after consideration of a second means of introducing CO₂ standards.

Admission of CO₂ from auxiliary mixing volume. The mixing volume [Fig. 2(a)] attached to the second vitreous silica inlet leak provided an alternative source of CO₂ for use in calibration. This method avoids the expense and complexity of the variable-volume inlet. Moreover, no control or replenishment is required to provide a CO₂ stream of constant strength, and CO₂ levels can be easily adjusted by regulation of gas flows within the mixing chamber. As mentioned in the experimental section, CO₂ can be added to the mixing volume as pure CO₂ or as a dilute mixture of CO₂ with helium. The latter approach is more convenient because the generation and control of ultra-low flow rates (i.e. the input flow of pure CO₂) is not required and because problems associated

with the presence of liquid CO₂ in tanks of pure CO₂ are avoided. Here, 0.7% CO₂ in He was adopted; a 44 liter cylinder lasts years even at constant flow. When a tank of pure CO₂ was used in various schemes (with a additional stage of helium dilution, with splitting of the CO₂ flow, etc.), the isotopic composition of the CO₂ changed due to continuous reequilibration of CO₂ between isotopically distinct liquid and gas phases within the tank. The drift amounted to $\approx +2\%$ (δ increased) as the tank pressure declined from 830 psig (initial charge) to 500 psig (complete evaporation of liquid). Further changes, to exhaustion of the supply, were $\approx +0.4\%$. For the CO₂-in-He mixture, the total variation thus far has been less than 0.1% and there has been no monotonic trend.

This technique was also applied to calibrate isotopic compositions of individual *n*-alkanes in the hydrocarbon mixture and, in this case, the 10-kV instrument was used. Under the conditions specified in the experimental section, amounts of CO₂ reaching the ion source were $10\times$ smaller than with the 3-kV instrument but, due to the higher sensitivity, integrated ion currents were about three-fold greater. As expected (based on the three-fold larger signal), the overall performance was $3^{1/2}\times$ better, the rms Δ ranging from 0.06 to 0.08%. The average Δ was 0.03% and there was, thus, no evidence for any systematic error associated with the use of the mixing volume.

Discussion. For the 10-kV instrument only, an artifact was observed when either the variable-volume inlet or the mixing volume was used to superpose square peaks of CO₂ on effluent streams containing background CO₂ concentrations yielding *m/z*-44 ion currents greater than 0.3 nA. Specifically, average values of Δ as large as 0.5% were observed (indicating a systematic error) *only* when the CO₂ calibrating gas was depleted in ¹³C relative to the background CO₂. No similar error was observed when *gaussian* peaks of CO₂ of any isotopic composition were employed as standards. This artifact is very easily avoided—by use of standard CO₂ that is enriched in ¹³C relative to the background or, more generally, by the effluent-diversion techniques discussed below—and its origins have proven obscure.

The fully differential procedure of isotopic calibration, i.e. that in which organic compounds with characteristics similar to the analytes were cojected, cochromatographed, and combusted along with the analytes, offered better performance when the system was not operating ideally. Specifically, when combus-

tion was incomplete, systematic errors were reduced by use of coinjected standards. This certainly does not mean that incomplete combustion should ever be tolerated, but the phenomenon is of interest because it provides a view of the combustion process.

In experiments in which NiO was used in the combustion reactor as the only oxygen donor and catalyst (n.b. not even minor amounts of CuO were present and no O_2 was added to the gas stream, a non-standard operating procedure used only in studies of combustion processes), incomplete combustion was observed at temperatures between 900–1000°C (Merritt *et al.*, 1994), especially when demands for O_2 were high and continuous due to bleeding of the liquid phase at high column temperatures. As shown in Fig. 5(a) and (b), values of Δ for calibration based on the coinjected organic standards were then very much smaller than those for calibration based on CO_2 .

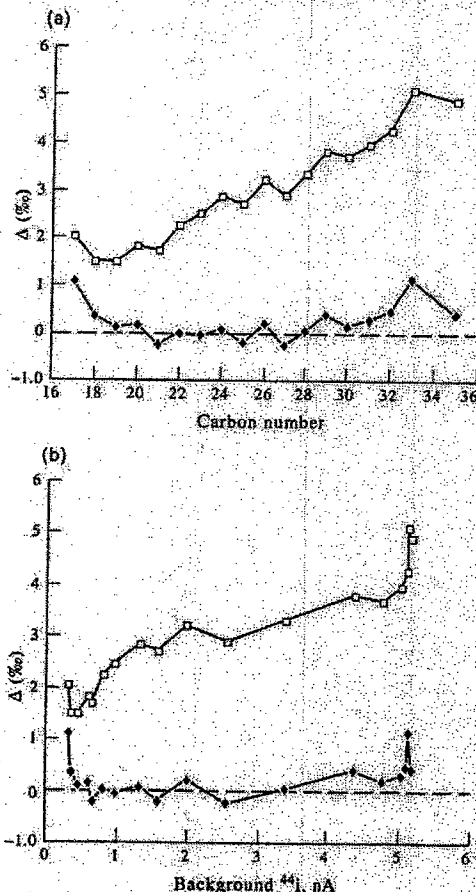


Fig. 5. Values of Δ as a function of (a) carbon number in homologous series of n -alkane test samples and (b) background CO_2 level (due to combustion of liquid phase bleeding from the chromatographic column at high temperatures). Combustion was incomplete (see text) and this is sharply reflected by the difference in δ values based on coinjected deuterocarbons (solid diamonds) and CO_2 standards (open squares).

standards. Presumably this occurred because the same systematic errors affected both the analytes and the coinjected organic standards. For values of δ based on the CO_2 -gas standards, the roughly linear relationship between the systematic error and the background CO_2 level [Fig. 5(b)] suggests that incompleteness of combustion is exacerbated by competition for O_2 . Even though use of the coinjected standards almost neutralized the systematic error, overall performance was still significantly degraded, with the rms Δ increasing to 0.4‰ (vs 0.07‰ for the same system under conditions allowing quantitative combustion). Similar but less severe errors have been observed when CuO-based combustion reactors failed due to exhaustion of their O_2 -donor capacity (which can be detected by distortion of the 45/44 ratio signal, notably the appearance of a second, broad, positive deflection following the negative deflection; by a decrease in the expected yield of CO_2 ; and by disappearance of these same faults when the reactor is reoxidized). Typical results are shown in Fig. 6.

Introduction of isotopic standards while diverting the effluent stream

Whatever its strengths, the coinjected-standard procedure lacks flexibility because a considerable variety of standards must be maintained if problems with coelution are to be minimized. Coelution of standards with sample components has been observed to cause systematic errors larger than 1‰ even when overlaps appeared minor (Lichtfouse *et al.*, 1991; Collister, 1992; Ricci *et al.*, 1994). Whenever a standard peak has been corrupted and not excluded from the calibration, analyses of all peaks for which assignments of δ have been based at least partly on that standard (see Ricci *et al.*, 1994) will be inaccurate. This is, however, only one form of a general problem that can affect any calibration scheme in which standards are superposed on the sample stream. Peaks of CO_2 from the variable-volume inlet or the mixing volume can be corrupted by sample components that appear unexpectedly or which are too small to be noticed readily. In many chromatograms, it is impossible to find an interval of even a few seconds in which the baseline is free of peaks. Even with complete control of CO_2 -injection times, insertion of reliable standards is then impossible. Security of standards against corruption by CO_2 from sample components can be guaranteed only if the effluent stream is diverted while standards are being observed.

Additionally, even when interference is avoided, signal-to-background ratios for standards decrease as background levels of CO_2 increase (e.g. due to column bleed). As a result, the precision of background-corrected ion-current and ratio measurements for standards decreases (Merritt and Hayes, 1994). Since performance is ultimately limited by the precision of measurements of standards, this factor

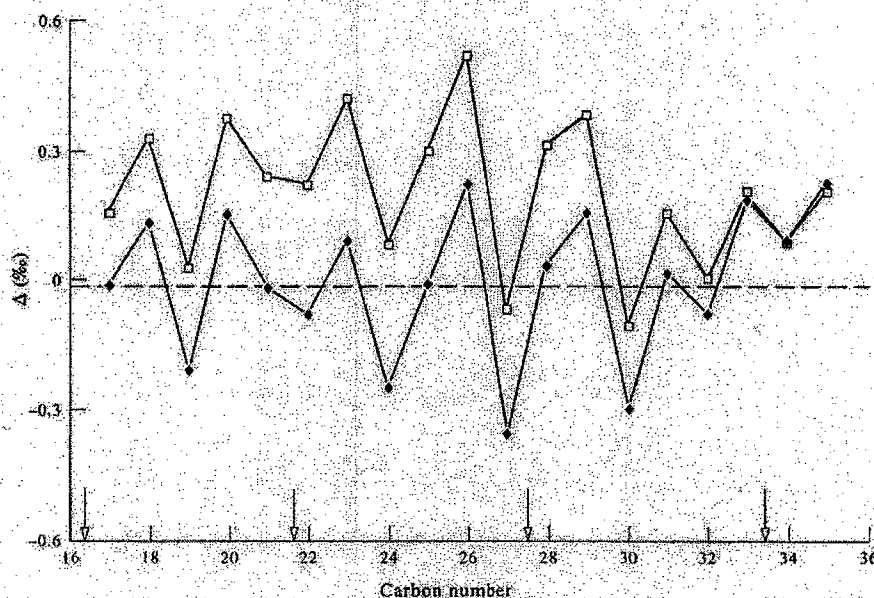


Fig. 6. Values of Δ for trial analysis of *n*-alkane test mixture when O_2 -donor capacity of CuO in combustion furnace was nearing exhaustion. Precision is severely degraded for both deuterocarbon- (filled solid) and CO_2 -based (open squares) calibrations, but systematic errors are much larger in the CO_2 -based data set.

also favors diversion of the effluent stream in order to reduce background levels of CO_2 .

The diversion must furnish a constant baseline (most easily guaranteed by quantitative diversion) and must be accomplished without altering the performance or characteristics of any part of the sample pathway, particularly the ion source. To maintain constant conditions in the ion source, the helium stream carrying the chromatographic effluent must be replaced and, to minimize loss of information while the effluent is diverted, all transitions must be as rapid as possible.

Efficiency of effluent diversion: Two methods were employed: (i) the device shown in Fig. 2(b) was used to remove the capillary leak from the effluent stream or (ii) valves B and C were toggled to change gas pathways. In both cases, diversion occurred downstream from the water separator so that the gas stream being manipulated was dry and minimally susceptible to sorption-desorption artifacts. Recordings of ion currents during effluent diversion at three different effluent- CO_2 levels are shown in Fig. 7. The instants at which the capillary was removed from or reinserted in the effluent stream are marked by arrows. In each case, the signal responds about four seconds after the command, this delay representing the transit time for gas in the capillary inlet leak. When diversion was accomplished by toggling valves B and C, the lag was about one second longer, presumably because reversals of flow required at least small buildups of pressure. Both techniques diverted the effluent stream with equal efficiency. Despite its

greater speed, the capillary-retraction device had several disadvantages. First, because the capillary leak was physically removed from the inlet capillary by only a small distance, a small portion of the effluent stream could be transmitted to the mass spectrometer if the auxiliary helium stream in the device was not strong enough to deflect effluent stream completely. Second, depending on its rigidity (which seemed to vary) and the precision of physical alignment, the moving capillary could fail to enter the transfer capillary without making physical contact. Fractures could occur, or the capillary leak might simply bend enough to accommodate the movement of the piston without actually entering the transfer capillary. For these reasons, the alternative diversion scheme was preferred unless timing was critical.

For both devices, ion currents at first dropped off at rates governed by the time constants of the electrometers. The electronic baseline was, however, not reached immediately. As shown in Fig. 7, small but detectable residual ion currents slowly declined over the time interval prior to introduction of the CO_2 standard (here, to show the decline clearly, that interval was extended to 40 s). Specifically, ion currents prevailing 5 s after the major drop in the signal are <2% of the effluent CO_2 level and the 45/44 ratio for the residual currents changes by <5% during the next 60 s. Because the magnitudes of those residual ion currents were proportional to the CO_2 levels prevailing in the effluent at the moment of its diversion, we assume that they derive from traces of CO_2 .

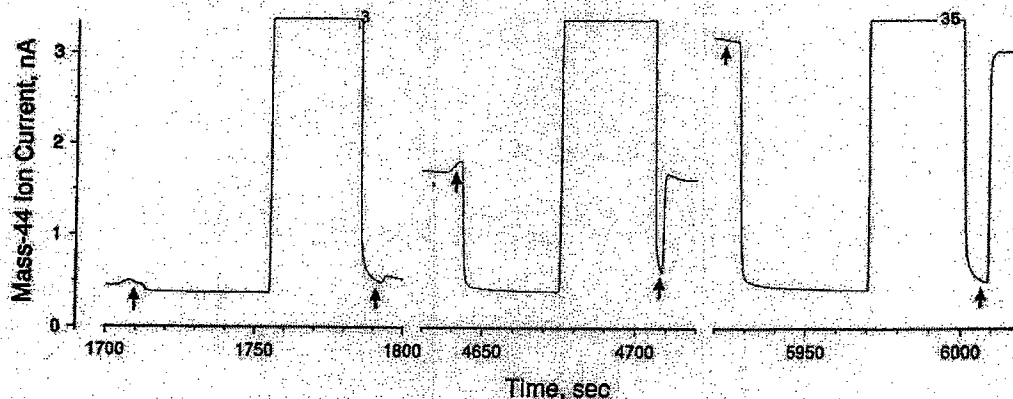


Fig. 7. High resolution plots of the m/z -44 baseline during diversion events produced by the device shown in Fig. 2(b). For each time interval shown, the first arrow on the trace marks the time at which the MS-inlet capillary was withdrawn from the effluent stream and the second arrow marks the time of its reinsertion. The nearly complete chromatographic run is shown in Fig. 8.

sorbed and slowly released along the pathway between the diverter and the ion source. Neither device is superior to the other in terms of completeness of diversion.

The indicated residual ion currents represent the background against which isotopic standards will be observed. As shown in Fig. 8, signals produced by standard gases are commonly ≈ 4 V, corresponding to an ion current of 13 nA. A residual ion current corresponding to 1% of a 3-nA background would

thus comprise 0.2% of the standard signal. The accuracy of the isotopic calibration will be affected if the isotopic composition of the residual gas differs significantly from that of the standard and if no correction is applied for its presence. For example, if the δ values of the standard and the residual gas differed by 30% and the residual gas contributed 0.2% of the signal, the resulting error in δ values assigned to samples would be 0.07% (this is a plausible worst case; although residual currents amount to

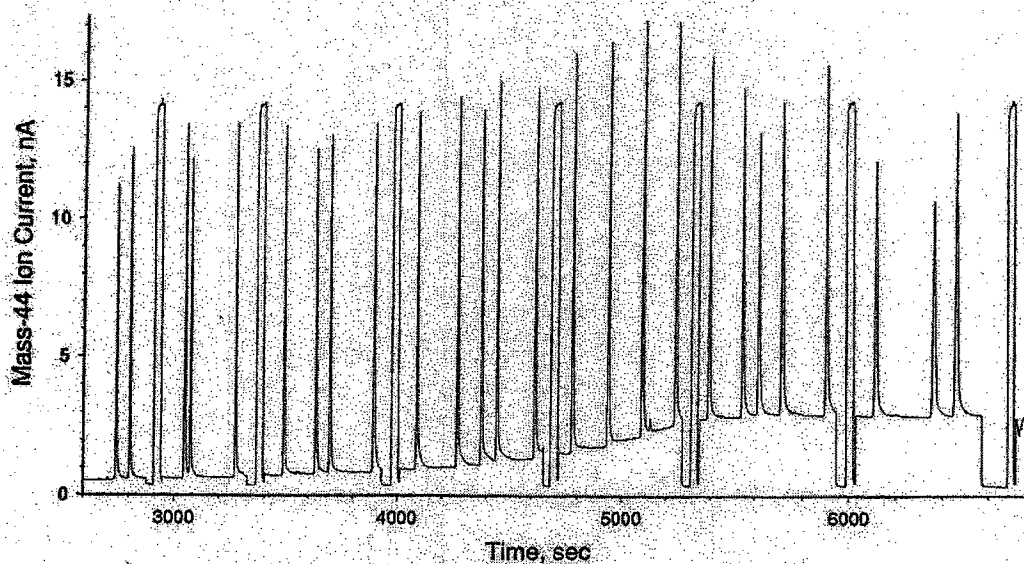


Fig. 8. Ion-current signal (m/z 44) for run in which the effluent stream was diverted when CO_2 standards were introduced from the CO_2 mixing chamber (see also Fig. 7). Note that, since they are not superposed on the chromatographic background, all CO_2 -standard peaks rise to the same signal height. These data were generated using the 10-kV instrument. Chromatographic conditions and identifications of peaks as for Fig. 3.

$\approx 2\%$ of the effluent CO_2 level 5 s after diversion, they are still declining and 1% is a reasonable estimate for the integrated contribution over the width of a 20-s standard peak).

Correction for effects of residual currents: Because such offsets would be at the threshold of detection and possibly responsible for limiting the accuracy of the technique, we examined two alternative strategies for generation of calibration data. In each case, standard CO_2 was introduced from the mixing volume when the capillary leak was physically retracted from the effluent stream. For the trials exemplified by Figs 7 and 8, admission of standard was delayed for 30–40 s after diversion in order to allow maximal decay and precise observation of residual ion currents. For example, for the standard peaks shown in Fig. 8, residual signals were observed at retention times of approx. 1752, 4674, and 5968 s. Ion-current ratios related to standards were corrected for effects of these individually measured residual currents. In the trials exemplified by Fig. 9, standard CO_2 was admitted at the same instant that the effluent was diverted. Signals related to standards were corrected only by subtraction of an electronic baseline signal observed during the last 30 s of the backflush period at the beginning of each chromatogram. Together, these trials represent best- and worst-case extremes. The 30–40 s decay interval would, in many practical cases, represent an extravagant disposal of observation time, but was chosen here to allow precise observation of minimal residual currents. At the other extreme, complete neglect of residual currents is not necessary since an algorithm could be developed in which it was assumed that the residual

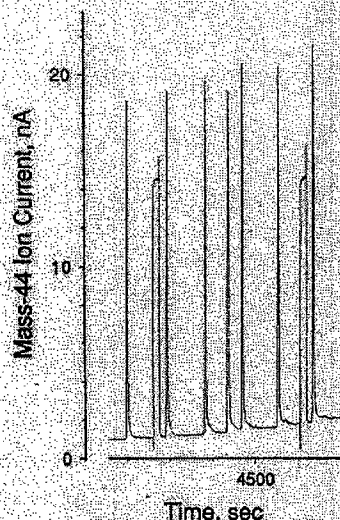


Fig. 9. Ion-current signal (m/z 44) for a portion of a chromatogram identical to that shown in Fig. 8 except that diversion of the effluent stream and introduction of CO_2 standards were simultaneous.

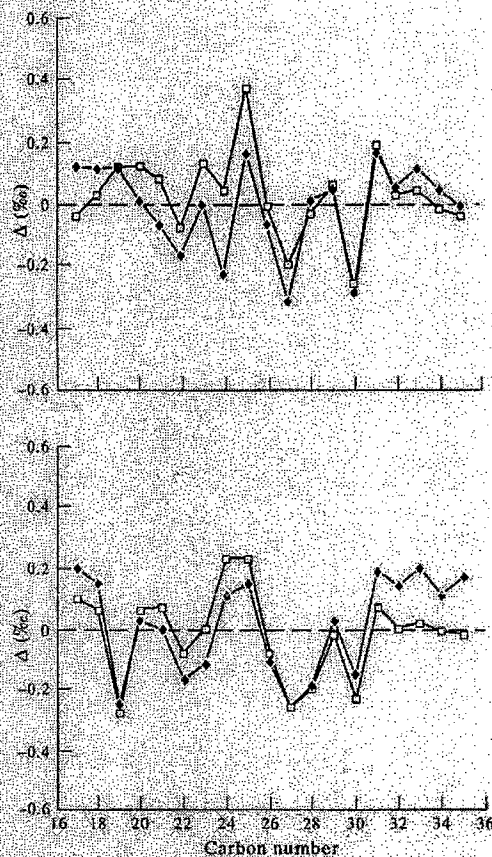


Fig. 10. Values of Δ for analyses like that shown in Fig. 8. Symbols denote type of standard employed for isotopic calibration: open squares, CO_2 ; solid diamonds, deuterocarbons. Gas standards were isotopically depleted (a, $\delta = -44.5\%$) and isotopically enriched (b, $\delta = +25.1\%$) by 9–10% relative to combusted column bleed.

current beneath each standard amounted to a specified fraction of the immediately preceding effluent CO_2 level.

When residual currents were observed individually and specific corrections applied to standard ion currents (Figs 7 and 8), average Δ values ranged from -0.03 to $+0.03\%$ and rms Δ values ranged from 0.12 to 0.14%. Individual Δ values for representative chromatograms are graphically summarized in Fig. 10(a) and (b), which reflect trials with two different CO_2 standards, one enriched, the other depleted in ^{13}C relative to sample and background C. The same chromatograms included co-injected deuterioalkanes, and Δ values obtained for calibrations based on those standards are also shown. Similar accuracy and precision were obtained in additional trials (not shown) in which (i) the period allowed for decay and observation of residual currents was shortened to 10 s, and (ii) the effluent stream was diverted by changing gas-flow pathways rather than by physically removing the restrictor capillary from the transfer capillary.

When standard introduction and effluent-stream diversion occurred simultaneously (Fig. 9), average values of Δ again ranged from -0.03 to $+0.03\%$ and rms Δ values increased slightly, to a maximum of 0.16% . In this case, the first 5 s of each standard peak were ignored in order to minimize effects of residual currents.

CONCLUDING REMARKS

The performance of calibration techniques involving diversion was, in these tests with homologous series of easily and widely resolved substances, slightly lower than that obtained when effluent diversion was avoided. A straightforward conclusion follows: to obtain maximal performance in the rare instances in which chromatograms provide adequate "flat spots" allowing superposition of standards on the effluent stream, avoid diversion. In general, however, samples of interest yield chromatograms of such complexity that diversion furnishes the only practical means of calibration. Overlap between standards and sample components will cause serious systematic errors, not only for the sample component that happens to interfere but for all δ values based on the overlapped standard. Such errors can be securely avoided only by use of diversion.

Additionally, extended practical experience suggests that the differences between calibration procedures may not be as great as those suggested by these specific trials. In an effort to test this, we have surveyed hundreds of chromatograms in which assessment of accuracy and precision is possible. Results are summarized in Table 1. Most importantly, no average Δ value differs significantly from zero—there is no evidence that any of these procedures yields results that are systematically in error. Notably, the rms Δ values of the no-diversion measurements ranged more widely than those incorporating diversion-based calibrations. It appears that avoidance of diversion can furnish highest performance but that, in many cases where greatest care for optimization is not taken, the performance is no better than that of diversion-based techniques.

Acknowledgements—We are indebted to Katherine H. Freeman, Margaret P. Ricci, and Stephen A. Studley for valuable discussions and technical assistance. We thank Martin Schoell and Robert Carlson of Chevron Petroleum Technology Co. for the homologous series of *n*-alkanes and the perdeuterated *n*-alkane standards that were used in these studies. We also thank Finnigan MAT for making available the MAT 252 and Chevron and MAT for supporting purchase of the Delta S and development of irmGCMS. Partial support of personnel costs associated with this work has come from NASA (NAGW-1940) and from a GE Fellowship awarded to D.A.M.

REFERENCES

- Barrie A., Bricout J. and Koziol J. (1984) Gas chromatography-stable isotope ratio analysis at natural abundance levels. *Biomed. Environ. Mass Spectrom.* **11**, 583–588.
- Collister J. W. (1992) An isotopic biogeochemical study of the Green River Oil Shale (Piceance Creek Basin, Colorado). Ph.D. Thesis, Department of Geological Sciences, Indiana University, Bloomington, Ind., U.S.A.
- Frazer J. W. and Crawford R. W. (1963) Modifications in the simultaneous determination of carbon, hydrogen, and nitrogen. *Mikrochim. Acta* 561–556.
- Freedman P. A., Gillyon E. C. P. and Jumeau E. J. (1988) Design and application of a new instrument for GC-isotope ratio MS. *Am. Lab.* **20**, 114–119.
- Hayes J. M., Freeman K. H., Popp B. N. and Hoham C. H. (1990) Compound-specific isotopic analyses: a novel tool for reconstruction of ancient biogeochemical processes. In *Advances in Organic Geochemistry 1989* (Edited by Durand B. and Béhar F.). *Org. Geochem.* **16**, 1115–1128. Pergamon Press, Oxford.
- Lichtfouse E., Freeman K. H., Collister J. W. and Merritt D. A. (1991) Enhanced resolution of organic compounds from sediments by isotopic gas chromatography. *J. Chromatogr.* **585**, 177–180.
- Merritt D. A., Freeman K. H., Ricci M. P., Studley S. A. and Hayes J. M. (1994) Performance and optimization of a combustion interface for isotope-ratio-monitoring GCMS. Submitted to *Anal. Chem.*
- Merritt D. A. and Hayes J. M. (1994) Isotope-ratio-monitoring mass spectrometry. Submitted to *Anal. Chem.*
- Ricci M. P., Merritt D. A., Freeman K. H. and Hayes J. M. (1994) Acquisition and processing of data for isotope-ratio-monitoring mass spectrometry. *Org. Geochem.* **21**, 561–571.
- Santrock J., Studley S. A. and Hayes J. M. (1985) Isotopic analyses based on the mass spectrum of carbon dioxide. *Anal. Chem.* **57**, 1444–1448.

L'EQUIPE

Landis archi-positif !

DAMIEN RESSIOT

867 words

24 April 2007

L'Équipe

3

French

Copyright 2007 L'Equipe "All Rights Reserved"

CYCLISME

DOPAGE

Landis archi-positif !

Les analyses rétrospectives sont claires : l'Américain était plusieurs fois positif à la testostérone sur le Tour 2006.

Il n'y a désormais plus aucun doute : **Floyd Landis** a bel et bien triché pour remporter le Tour de France 2006. Contrôlé positif à l'issue de

la 17 étape, l'Américain

a depuis toujours nié

s'être dopé en espérant échapper à toute sanction pour vice

de procédure. Mais les analyses rétroactives

de sept échantillons urinaires prélevés pendant la Grande Boucle ont confirmé

une prise exogène

de testostérone.

CONTRÔLE POSITIF à la testostérone une première fois, à l'issue de la 17 étape du dernier Tour de France, **Floyd Landis**, vainqueur en sursis, va devoir désormais réorienter sérieusement sa stratégie de défense. En effet, ce n'est pas une, mais plusieurs infractions aux règles antidopage que le trentenaire américain a commises en juillet 2006, puisque plusieurs de ses échantillons prélevés lors de cette même épreuve contiennent des traces de testostérone synthétique...

Après s'être opposé vigoureusement à l'idée même d'analyses complémentaires menées par le laboratoire de Châtenay-Malabry (LNDD), l'ancien leader de Phonak avait dû finalement se résoudre à cette extrémité, en vertu d'une décision prononcée par le panel (AAA) de l'Agence américaine antidopage (USADA). On comprend désormais mieux son appréhension depuis que l'on connaît le résultat des analyses supplémentaires, lesquelles ont duré une semaine en raison de la complexité de la méthode utilisée, la spectrométrie de masse (IRMS, voir ci-dessous).

Cette méthodologie, qui permet de distinguer la présence de testostérone naturelle – fabriquée par l'organisme – de celle produite par synthèse, livre en effet des résultats irréfutables et n'aboutit jamais à la mise en cause de faux positifs. Parmi les sept échantillons restants, analysés une nouvelle fois, plusieurs sont positifs.

Comment dès lors expliquer que des échantillons déclarés négatifs dans un premier temps puissent être déclarés positifs après de nouvelles analyses ? Tout simplement parce que, dans le cadre de la détection de routine de la testostérone, les laboratoires débutent le processus de l'analyse par l'établissement du célèbre ratio testostérone sur épitestostérone (T/E). Ce n'est que lorsque celui-ci est supérieur à 4, en vertu des

règles édictées par l'Agence mondiale antidopage, que les laboratoires commencent alors une instruction plus poussée, laquelle passe, lorsqu'ils sont équipés du matériel adéquat, par l'utilisation de la méthode IRMS. Lors du Tour de France 2006, un seul des échantillons appartenant à Landis dépassait la barre fatidique des 4 (11, en l'occurrence, comme seuil de confirmation). Les sept autres échantillons prélevés sur l'Américain étaient en revanche en deçà du seuil d'instruction et n'ont pas fait l'objet de recherche par IRMS. C'est cette nouvelle opération analytique qui a été menée à Châtenay-Malabry ces derniers jours.

Les droits

de la défense

respectés

Landis pourra-t-il, dès lors, comme il ne cesse de le faire depuis l'annonce de son premier contrôle positif, remettre en cause la crédibilité et la compétence des techniciens du LNDD, lui qui avait demandé, après s'être résigné au verdict du panel, que ces fameuses analyses rétrospectives soient effectuées au sein du laboratoire de Los Angeles (UCLA) ? Difficile.

En effet, les analyses supplémentaires opérées en France ont été menées dans le cadre de règles strictes, en aveugle (avec de nouveaux codes), avec l'adjonction d'échantillons supplémentaires (des leurres destinés à rendre toute identification aléatoire) et en présence de plusieurs experts représentant les deux parties, l'USADA et Landis (voir ci-dessous). Cette dernière précaution, imposée par l'Agence française de lutte contre le dopage (AFLD), s'est révélée extrêmement judicieuse pour préserver l'aspect contradictoire de l'expertise, et le coureur et sa cohorte d'avocats auront bien du mal à remettre en cause un processus qu'ils ont pu observer de très près. Nul doute en effet que les deux personnes présentes à Châtenay-Malabry durant les sept jours d'analyses – un avocat et un scientifique – auraient stoppé le processus ou crié au scandale si une irrégularité s'était produite au sein du laboratoire.

Bien entendu, ils ne devraient néanmoins pas renoncer à stigmatiser telle ou telle insuffisance, mais l'USADA, dont les deux représentants ont quitté la France dès samedi soir dernier, ont semblé totalement satisfaits par la manière dont les opérations se sont déroulées.

Désormais, et alors que cette même agence américaine s'intéresse également au financement de la campagne de défense de Landis, on attend avec curiosité la tonalité que ce dernier adoptera le 14 mai à Los Angeles, jour de sa comparution devant le panel de l'USADA.

Les débats, qui à l'heure actuelle sont évalués à une dizaine de jours, seront-ils publics, comme Landis l'avait expressément demandé ? Ce dernier, face à la nouvelle consistance des accusations, se ravisera-t-il et optera-t-il plutôt pour une négociation intelligente ? La seule certitude, pour l'heure, c'est que l'Américain, qui s'est engagé auprès des instances françaises ad hoc (AFLD) à ne pas courir en France en 2007, pourrait également s'abstenir en 2008. Suspension oblige.

200704241415166

Document EQUIPE0020070424e34o0001n

Landis Fails Drug Test After Triumph in Tour de France

Alessandro Trovati/Associated Press

After Floyd Landis pulled away on a 9.2-mile climb, the tour's director called it "the best stage I have ever followed."

By JULIET MACUR

Published: July 27, 2006

Floyd Landis, who on Sunday became the third American cyclist to win the Tour de France, tested positive for illegally high levels of testosterone during the race, his team announced today.

If a second test confirms the results, Landis may be stripped of his Tour de France championship. That has happened only once before, when Maurice Garin was disqualified in 1904 for breaking race rules. The Phonak team said that it had suspended Landis and that it would fire him if a backup test confirmed the initial result.

In a statement on its Web site, Phonak said the International Cycling Union had notified the team on Wednesday that tests on Landis's urine sample discovered "an unusual level of testosterone/epitestosterone ratio" after Stage 17 of the Tour de France. The team and the rider were "totally surprised," the statement said, and will ask for an analysis of a second sample taken at the same time "to prove either that this result is coming from a natural process or that this is resulting from a mistake."

In Stage 17, Landis produced one of the greatest performances in cycling history, improbably roaring back into the lead pack after falling eight minutes behind the day before. His subsequent victory became the feel-good story that the Tour needed, particularly after the way the race had begun.

On the eve of the Tour, three race favorites, including Jan Ullrich of Germany and Ivan Basso of Italy, and six other riders were kicked out of competition for being involved in a doping scandal in Spain. Four of the top five finishers behind Lance Armstrong when he captured his seventh consecutive Tour title in 2005 were out of the race. A total of 58 cyclists and others connected to cycling have been implicated in that doping ring.

Landis, raised in a Mennonite family from Pennsylvania, turned attention at the Tour away from the scandal and toward his performance. He has a degenerative hip condition and planned to have hip-replacement surgery this fall, and he spoke about his efforts to ride through the pain.

Gritting his teeth, Landis pedaled down the Champs-Élysées on the final day to win his first Tour de France. He finished the three-week, 2,267-mile race nearly a full minute

ahead of Oscar Pereiro of Spain and more than a minute ahead of Andreas Klöden of Germany, in third place.

“I want to say thank you to everybody who kept believing, most of all my team,” Landis said after accepting the winner’s trophy, with the Arc de Triomphe behind him and the United States flag flying atop flagpole in front of him. “When things weren’t going so well, they kept fighting and never stopped believing.”

Greg LeMond, a three-time Tour de France winner and the first American to win the event, said he believed in Landis, too. LeMond watched the race from his home in Minnesota and said the race seemed cleaner than in past years because the wattage output of the riders was not as high. He said the Spanish doping scandal had helped clean up the race.

But when LeMond heard rumors on Wednesday that Landis had tested positive, then on Thursday when he heard confirmation, he said he was “devastated.”

“It’s going to be such a blow to American cycling,” LeMond said. “But it’s a wake-up call about the sport, for sure.”

He added: “If there’s any positive outcome on this, I hope, for the sport’s sake, that Floyd has the courage to come clean on everything. He needs to say everything about the sport, what’s happened in the past, what’s happening right now. We need to be transparent about it, so we can look at the sport and say, ‘How do we fix it?’ ”

The tests from the Tour are conducted at a French laboratory at Chatenay-Malabry. Each day, samples were taken from the stage winner, the overall leader and three random riders.

Pat McQuaid, the president of U.C.I. said it would be “a great disappointment and an unacceptable violation” should the second sample come back positive. But he said it would also prove the effectiveness of U.C.I.’s antidoping tests.

Dick Pound, chairman of the World Anti-Doping Agency, said there would still be a “huge black mark” on the sport, regardless of the backup test result.

“For this to happen in your marquee event, that’s a stunning indictment of the state of the sport,” Pound said. “They have a huge problem, a really serious problem, but first they have to recognize it. It’s like an alcoholic. Unless you acknowledge you have a problem, it’s very hard to move toward a solution.”

Landis’s initial positive test result is far from the first time a doping scandal has clouded the sport. In the late 1960’s, the cyclist Tommy Simpson died of a heart attack and was found with amphetamines in his pocket. In 1998, the entire Festina team was kicked out after a huge stash of performance-enhancing drug paraphernalia was found in a team car.

Cyclists have used several methods to enhance their performances, including blood transfusions to pack their bodies with more oxygen-carrying red blood cells and EPO, which boosts endurance.

Testosterone, an anabolic steroid, is used for strength and endurance, and also for quicker recovery, said Donald Catlin, who runs the Olympic drug testing laboratory at U.C.L.A. Its use raises a rider's ratio of testosterone to epitestosterone.

Catlin said that the test to discover high levels of testosterone is two-pronged and labor-intensive. The first part, to see if there is a high t/e ratio, can take anywhere from 8 to 12 hours, he said. The second part, to see whether that high ratio comes naturally or from an external source, is also lengthy.

"This is not a slam-dunk case," he said of Landis's case. "There is work to do, and if there's ever a test that won't repeat a positive, it will be a really complex analysis, and this is one of them."

He also said that if Landis had a naturally high level of testosterone, someone would already know it or someone would be "running around finding the past results and plotting them on graph paper" to prove his innocence.

"These data are saved and someone can look them up," he said.

Though Landis was granted a waiver by the U.C.I. to receive cortisone shots before the Tour began, cortisone use would not result in a higher t/e level, Catlin said. (Cortisone is otherwise banned.)

Landis failed to show up today for a one-day exhibition race in Denmark, a day after he missed a similar race in the Netherlands. He competed, and won, a race in Stiphout, the Netherlands, on Tuesday. Attempts to reach him on his cellphone were unsuccessful, and the voice mailbox for the phone was full beginning late Wednesday night. Calls to some of his teammates have not been returned.

Landis's mother, Arlene, said this morning that she heard from her son on Tuesday but that the positive drug test was not discussed.

"I didn't talk to him about that, but I know he's taking medicine for the pain in his hip," she said. "They stirred up trouble for Lance, too."

http://www.boston.com/sports/other_sports/cycling/articles/2006/07/30/lemond_landis_could_be_symbol_of_change/

LeMond: Landis could be 'symbol of change'

By Associated Press | July 30, 2006

PARIS -- Three-time Tour de France champion Greg LeMond says the doping charges against fellow American Floyd Landis could be "what cycling has needed for many years" in order to discourage cheating.

"If he is confirmed positive, I hope he has the courage to tell the truth," LeMond said in an interview with French weekly *Le Journal du Dimanche* released yesterday. "He alone can change the face of the sport today. His example could be a symbol of change."

In a veiled reference to seven-time winner Lance Armstrong, long dogged by doping allegations, LeMond added: "I hope that [Landis] won't do what another American did: deny, deny, deny."

LeMond, who won cycling's premier event in 1986, '89, and '90, urged a mass crackdown on doping, involving all cycling regulatory bodies, governments, and health ministries. He suggested an amnesty for riders who speak out about doping.

"Testing, we will never do enough of it," he said, urging the International Cycling Union -- which he has criticized as corrupt -- to take a more aggressive stance on doping.

Landis's victory ride into Paris last Sunday after a stunning comeback renewed enthusiasm for the sport -- but just days later, his title was in doubt after a French lab reported a testosterone imbalance in one of his urine tests.

Landis has said his body's natural metabolism was to blame and demanded backup tests.

Landis Fails Backup Test; Tour Title in Jeopardy

Pierre-Philippe Marcou

Weekend Edition Saturday, August 5, 2006 · A follow-up test on cyclist Floyd Landis has also shown high levels of testosterone. The cycling team Phonak fired him and he may lose his Tour de France title, but he vows to fight to clear his name. Charles Pelkey, an editor for *VeloNews*, talks with Scott Simon about the morning's news.

PARIS (AP) -- Floyd Landis was fired by his team and the Tour de France no longer considered him its champion Saturday after his second doping sample tested positive for higher-than-allowed levels of testosterone.

The head of France's anti-doping commission said the samples contained synthetic testosterone, indicating that it came from an outside source.

The second or "B" sample, "confirmed the result of an adverse analytical finding" in last week's "A" sample, the International Cycling Union said.

Pierre Bordry, who heads the French anti-doping council, said the lab that found higher-than-allowable levels of the hormone in both samples also discovered synthetic testosterone.

"I have received a text message from Chatenay-Malabry lab that indicates the 'B' sample of Floyd Landis' urine confirms testosterone was taken in an exogenous way," Bordry told The Associated Press.

Landis had claimed the testosterone was "natural and produced by my own organism."

The Swiss-based team Phonak immediately severed ties with Landis and the UCI said it would ask USA Cycling to open disciplinary proceedings against him.

"Landis will be dismissed without notice for violating the teams internal Code of Ethics," Phonak said in a statement.

"Landis will continue to have legal options to contest the findings. However, this will be his personal affair, and the Phonak team will no longer be involved in that."

Tour de France director Christian Prudhomme said Landis no longer was considered champion, but the decision to strip him of his title rests with the UCI.

"It goes without saying that for us Floyd Landis is no longer the winner of the 2006 Tour de France," Prudhomme told The Associated Press in a telephone interview. "Our

determination is even stronger now to fight against doping and to defend this magnificent sport."

Prudhomme said runner-up Oscar Pereiro of Spain would be the likely new winner.

"We can't imagine a different outcome," Prudhomme said.

If stripped of the title, Landis would become the first winner in the 103-year history of cycling's premier race to lose his Tour crown over doping allegations.

UCI lawyer Philippe Verbiest said Landis would officially remain Tour champion pending the American disciplinary process.

"Until he is found guilty or admits guilt, he will keep the yellow jersey," he said. "This is normal. You are not sanctioned before you are found guilty."

If found guilty, Landis also faces a two-year ban from the sport.

Despite the second positive test, Landis maintained his innocence.

"I have never taken any banned substance, including testosterone," he said in a statement. "I was the strongest man at the Tour de France, and that is why I am the champion."

"I will fight these charges with the same determination and intensity that I bring to my training and racing. It is now my goal to clear my name and restore what I worked so hard to achieve."

Landis' urine sample was analyzed at the Chatenay-Malabry lab outside Paris.

The results of the second test come nearly two weeks after he stood atop the winner's podium on the Champs-Elysees in the champion's yellow jersey.

Landis' positive tests set off what could now be months of appeals and arguments by the American, who says the positive finding was due to naturally high testosterone levels. He has repeatedly declared his innocence.

"It's incredibly disappointing," three-time Tour winner Greg LeMond said by phone from the starting line at the Pan Mass Challenge in Sturbridge, Mass. "I don't think he has much chance at all to try to prove his innocence."

The tests were conducted on urine samples drawn July 20 after Landis' Stage 17 victory during a grueling Alpine leg, when he regained nearly eight minutes against then-leader Pereiro -- and went on to win the three-week race.

The case is expected to go to the U.S. Anti-Doping Agency; the process could take months, possibly with appeals to the Court of Arbitration for Sport.

"It doesn't end here," said Landis' Spanish lawyer, Jose Maria Buxeda. "What matters is the concept. A prohibited substance has been found in the samples, but no immediate sanction comes into effect yet. The rider will defend himself."

Landis, a 30-year-old former mountain biker, says he was tested eight other times during the three-week tour and those results came back negative.

Landis' spokesman Michael Henson confirmed this week that the rider had tested positive for a testosterone-epitestosterone ratio of 11:1 -- well above the 4:1 limit.

Landis has hired high-profile American lawyer Howard Jacobs, who has represented several athletes in doping cases.

Jacobs plans to go after the UCI for allegedly leaking information regarding the sample testing.

Earlier this week, a New York Times report cited a source from the UCI saying that a second analysis of Landis' "A" sample by carbon isotope ratio testing had detected synthetic testosterone -- meaning it was ingested.

Since the Phonak team was informed of the positive test on July 27, Landis and his defense team have offered varying explanations for the high testosterone reading -- including cortisone shots taken for pain in Landis' degenerating hip; drinking beer and whiskey the night before; thyroid medication; and his natural metabolism.

Another theory -- dehydration -- was rebuffed by anti-doping experts.

"When I heard it was synthetic hormone, it is almost impossible to be caused by natural events. It's kind of a downer," said LeMond, the first American to win the Tour. "I feel for Floyd's family. I hope Floyd will come clean on it and help the sport. We need to figure out how to clean the sport up, and we need the help of Floyd."

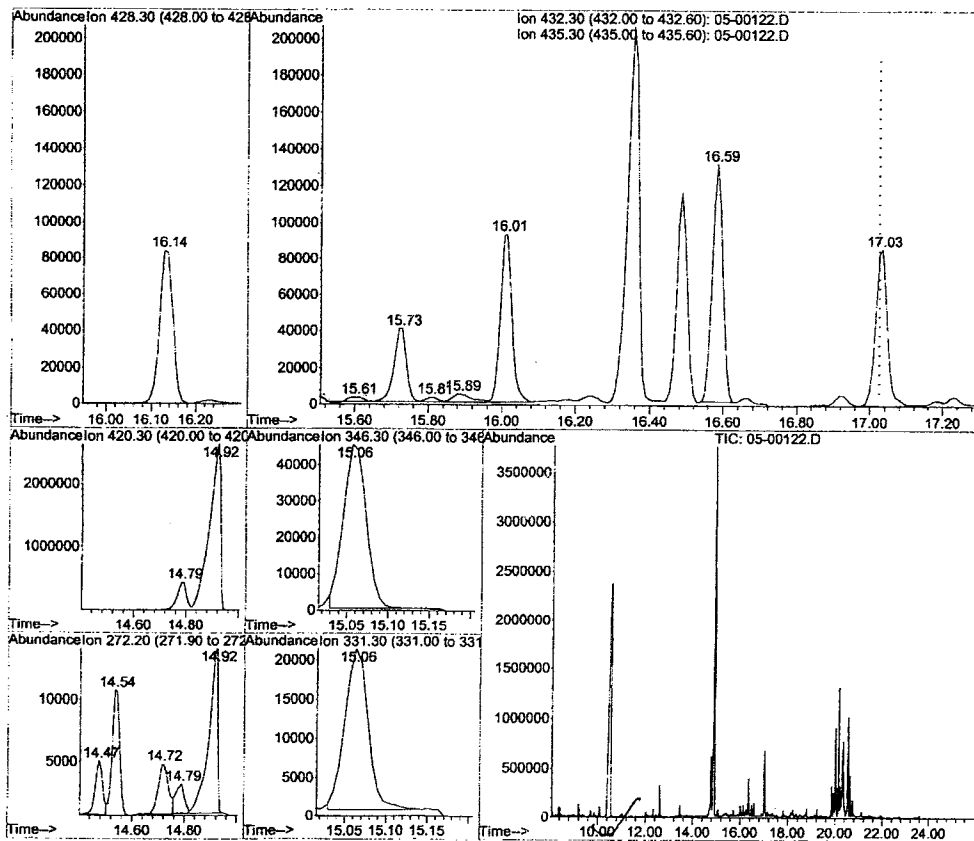
| 995474 | Original Result | Auto | Manual | Zero | Masslynx |
|-----------------|-----------------|-------|--------|-------|----------|
| A Sample | | | | | |
| E-11K | -2.58 | -1.72 | -2.32 | -1.76 | -2.18 |
| A-11K | -3.99 | -3.14 | -3.65 | -2.94 | -3.78 |
| 5B-P | -2.15 | -1.70 | -2.65 | -2.08 | -2.63 |
| 5A-P | -6.14 | -5.65 | -6.95 | -5.55 | -7.22 |
| B Sample | | | | | |
| E-11K | -2.02 | -0.32 | -0.35 | -1.66 | -2.39 |
| A-11K | -3.51 | -1.67 | -1.61 | -2.81 | -4.01 |
| 5B-P | -2.65 | -3.37 | -3.05 | -2.33 | -2.80 |
| 5A-P | -6.39 | -7.61 | -7.19 | -5.58 | -7.03 |

| Blanks | Original Result | Auto | Manual | Zero | Masslynx |
|-----------------|-----------------|-------|--------|-------|----------|
| A Sample | | | | | |
| E-11K | -0.87 | -0.51 | -0.56 | -0.06 | 0.09 |
| A-11K | -0.48 | -0.49 | -0.53 | -0.02 | -0.59 |
| 5B-P | -0.55 | -0.92 | -0.27 | -0.47 | -1.00 |
| 5A-P | -1.59 | -3.65 | -1.87 | -1.46 | -2.45 |
| B Sample | | | | | |
| E-11K | -1.08 | -1.11 | -0.94 | -0.25 | -0.51 |
| A-11K | -0.08 | 0.03 | 0.17 | 0.83 | 0.55 |
| 5B-P | -0.67 | -1.33 | -0.69 | -0.54 | -1.52 |
| 5A-P | -1.60 | -3.45 | -1.89 | -1.24 | -3.66 |

Rapport de quantification
Quantification Testostérone et Épitestostérone
Dernière modification: Mon Nov 15 10:35:39 2004

Fichier : M:\DATA\050120RC\G
Opérateur : CHRISTOPHE
Échantillon : 05-00122 dep apres HRMS
Info. suppl.: LOT P4 # 05-016 dep apres HRMS
Instrument : MS¹²
Vial no : 3
Analyse : 12:21 par P4SIM.M
Quantification: 12:47 par M:\METHODES\P4SIM.M
Multiplicateur: 33.33

| Produit | Signal | TR | Aire | Concentration |
|-------------------|------------|-------|---------|---------------|
| ISTD 2 | m/z 428.30 | 16.14 | 1705309 | |
| Testosterone | m/z 432.30 | 16.59 | 2509863 | 69.7 ng/mL |
| Epitestosterone | m/z 432.30 | 16.01 | 1986949 | 57.3 ng/mL |
| ratio T/E corrigé | | | | 1.38 |
| ISTD 3 | m/z 435.30 | 17.03 | 1857726 | |



Échantillon: 05-00122 dep apres HRMS

Page 1 de 4

PETER HEMMERSBACH, PH.D. WITNESS STATEMENT

I, Peter Hemmersbach, will state as follows:

1. Background

- 1.1. I have been engaged in doping control analysis since 1985 at the Hormone Laboratory at Aker University Hospital in Oslo, Norway. From 1991 I have been head of the IOC-accredited laboratory (from 2004 WADA-accredited) for doping control analysis.
- 1.2. I got my professional education in chemistry from the University in Münster, Germany, with a Dipl. Chemiker (comparable MSc) and Dr. rer. nat. (comparable PhD) degree.
- 1.3. From 2000 I have been appointed as Professor II for pharmaceutical and doping analysis at the University in Oslo, School of Pharmacy, Department for Pharmaceutical Chemistry, Norway.
- 1.4. From 1996 I have been member of the IOC Medical Commission, Subcommission for Doping and Biochemistry in Sport (from 2004 IOC Medical Commission Games Group).
- 1.5. From 2004 I have been member of WADA's Laboratory Committee. This committee is responsible, among other duties, for reviewing and updating the International Standard for Laboratories as well as the Technical Documents.
- 1.6. The proficiency testing of laboratories engaged in doping control has been determined by the IOC (until 2003) and now by WADA. The above mentioned committees provide advice to the decision-making bodies regarding laboratory recognition.
- 1.7. Since 1999 I have been working as a technical assessor for the national accreditation bodies in Norway, Denmark, Germany, Switzerland and Belgium. The audits I took part in were laboratory accreditations according to ISO 17025.
- 1.8. For several audits I have acted as technical assessor for the Swiss Accreditation Service (SAS). A part of the accreditation process evaluates the competence of the scientific staff to select, validate, and control new methods. During these audits I became familiar with the WADA-accredited laboratory in Lausanne. Although my main field of expertise is chromatographic separation methods with mass spectrometric detection (GC/LC-MS), I have had an opportunity to observe the commitment to and implementation of quality management systems, the good organization of the laboratory, the competence of the employees, and the positive attitude and motivation of the whole staff.

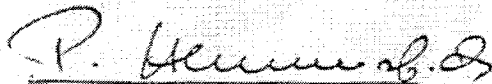
2. ISO and ISL Laboratory Accreditation

- 2.1. The WADA International Standard for Laboratories (ISL) was developed in part as an application of ISO 17025 to the specific needs of the field of doping control. WADA has worked with the International Laboratory Accreditation Cooperation (ILAC) to obtain recognition of the ISL and has trained ILAC

assessors in the use of the ISL in laboratory accreditation of doping control laboratories. Some countries have already adopted the ISL into their assessment visits, which clearly demonstrates the harmony between the ISL and ISO 17025. Thus the ISL is the relevant document for accreditation in doping control, just as the NCCLS EP12-A document referred to by Dr. Strong may be the relevant document for clinical laboratories.

- 2.2. The WADA ISL recognizes the necessity of both quantitative and qualitative (presence or absence) tests in doping control. The WADA Prohibited List prohibits the use of certain substances and methods. Therefore merely the presence of a doping agent, its metabolites or other markers in blood or urine may be proof of a doping offence. Only in the case of substances where endogenous production of the doping agent has to be taken into account is a threshold established. From a perspective of a laboratory measurement, the detection of two populations of red blood cells in a sample is not a physiological condition, and thus this is not a quantitative, threshold measurement.
- 2.3. A qualitative method is as objective as a quantitative approach, as long as the criteria for the presence of a second red blood cell population are adequate.
3. Method validation in qualitative methods
 - 3.1. Method validation is an important process that occurs simultaneously with method development.
 - 3.2. The term validation is used by different groups in different ways. From the laboratory perspective, validation is the confirmation that the method is fit for purpose. Validation is also used in some circles to refer to "proof of concept."
 - 3.3. Validation is carried out in the laboratory performing the test, in this case the LAD. Even if a standard procedure were transferred to the laboratory to follow, the laboratory would be required to carry out its own validation. The extent of the validation is determined by what information is available from previous studies and experience. From reviewing Dr. Saugy's testimony, the LAD did a validation study for the flow cytometry method.
 - 3.4. Validation for a qualitative test differs from a quantitative test. The WADA ISL discussed the validation of these two types of tests separately.
 - 3.5. From the perspective of the ISL and reviewing the written testimony of Dr. Saugy for this hearing, the laboratory appears to have established that no analytical false positives occur by determining that for several hundred known negative samples, the analytical system did not detect any false positive results.
4. Uncertainty in qualitative methods
 - 4.1. The WADA ISL notes that uncertainty, in a quantitative sense, cannot be applied to a qualitative method.

- 4.2. The process of determining uncertainty in qualitative methods is not developed to the extent that uncertainty in quantitative measurements has been.
- 4.3. Uncertainty in a laboratory setting refers to the ability of the analytical process to accurately measure a property of the sample. Some of the studies referred to in Dr. Saugy's testimony would provide an estimate of analytical uncertainty.
- 4.4. It is important in considering the potential for false positive or negative results that the detection capability of the method be considered. The existence of variation below the level of detection of the method would not result in false positives or negatives.



Peter J. Hemmersbach, Ph.D.

12.8.05

Date

Cyclist: Positive test really was negative

[Chicago Final Edition] *Chicago Tribune* - Chicago, Ill.

Author: Philip Hersh, Tribune Olympics reporter

Date: Oct 13, 2006

Section: Sports

Document Types: News Text

Word Count: 506

Taking the case to the public with a PowerPoint presentation and hundreds of pages of documents posted Thursday on his Web site, 2006 Tour de France winner Floyd Landis claimed his positive drug test actually was negative for scientific and secretarial reasons.

Confronted with the same evidence and a related motion for dismissal from Landis' lawyer, the U.S. Anti-Doping Agency's review board still decided Sept. 18 to charge the cyclist with doping based on a positive test for the banned steroid testosterone after the 17th stage of race.

Landis' attorney, Howard Jacobs, wrote off the USADA review as the equivalent of a kangaroo court, challenging its diligence and unwillingness to defy the higher authority of the World Anti-Doping Agency.

"Ask the review board how long they spent [on] it; I'm guessing it wasn't a lot," Jacobs said. "When (WADA chairman) Dick Pound says 'If USADA doesn't proceed, we will,' of course they [will] proceed."

Christiane Ayotte, director of the Montreal anti-doping lab, refuted those accusations. Ayotte also insisted Landis' defense team was inclined to have selected, without context, only pieces of evidence that could support their case from the files of the French laboratory that analyzed Landis' sample.

"WADA and USADA cannot act in a foolish matter," Ayotte said. "They have rules and lawyers. These cases must be based on scientific and legal matters, not political pressure."

The case is headed for an arbitration hearing early next year. Jacobs has asked the hearing be open.

"We are, obviously, not going to participate in a circus, but we have no objection to an open hearing," said USADA general counsel Travis Tygart.

Tygart declined to comment on specifics of the case. WADA spokesman Frederic Donze also declined comment.

The PowerPoint created by Arnie Baker, a retired San Diego physician identified as a longtime Landis coach and adviser, points out errors in recording information on laboratory forms; contends there is evidence of sample degradation and unacceptable variances in scientific measurements that should be sufficient to invalidate the positive result; and questions conclusions drawn from a carbon isotope ratio (CIR) test designed to see whether the testosterone in an athlete's sample is synthetic rather than naturally produced.

"[Landis' defense] must be able to cast doubt on the relevancy of the overall findings," Ayotte said. "A few boo-boos in hundreds of pages of documents is not enough to say the case goes into the wastebasket."

An athlete is presumed to have doped with testosterone if the ratio of two hormones, testosterone and epitestosterone, is greater than 4-to-1. The urine sample is divided into two parts; Landis' "A" sample had a ratio of 11.4-1 and the "B," 11-1.

Baker cited numbers related to contamination and variance in T/E levels that sounded "troubling" to a source familiar with doping control procedures.

Ayotte found it unlikely that degradation could have occurred in the two days between Landis' giving the urine sample and the lab's beginning analysis of it. She had not yet seen enough of the file to comment on measurement issues.

phersh@tribune.com

PRÉSENTATION
DU MAGAZINE

POUR ANNONCER
DANS LE MAGAZINE

jobboom.com

EMPLOIS GÉNÉRAL

EMPLOIS COMPTABILITÉ/
FINANCES

EMPLOIS GÉNIE

EMPLOIS SOUTIEN
ADMINISTRATIF

EMPLOIS TECHNOLOGIES
DE L'INFORMATION

EMPLOIS VENTES/
SERVICE À LA CLIENTÈLE

VOTRE CARRIÈRE


du 15 juin au
15 août 2005

[À la une]

Christiane Ayotte

La créativité en science

par Jean-Sébastien Marsan


Directrice du laboratoire de contrôle du dopage de l'Institut national de la recherche scientifique (INRS)-Institut Armand-Frappier, Christiane Ayotte use d'ingéniosité pour démasquer les stéroïdes anabolisants et autres drogues illégales en usage chez les athlètes professionnels.

«Je n'aborde jamais un projet de recherche sans avoir fait un état de la littérature scientifique. À cette étape, ce n'est pas l'intuition qui me caractérise. La créativité intervient dans l'établissement des hypothèses. «La partie créative et prospective de mes tâches, c'est de suivre la littérature scientifique, de regarder ce qui sort de l'industrie pharmaceutique et qui pourrait être détourné [en dopage clinique].»

La créativité dans la science, c'est faire preuve de curiosité multidisciplinaire. «Le défi scientifique en chimie est intéressant, mais ça ne me suffisait pas... J'avais un penchant pour la psychologie, notamment. Je dois penser comme un athlète qui se dope et comprendre le milieu du sport.»

La lutte contre le dopage a aussi des dimensions politiques, économiques, légales, etc., que Christiane Ayotte ne peut ignorer. «Quand des athlètes et de riches avocats américains se battent contre la validité des tests et des contrôles, on a intérêt à être créatif! Et comme ce sont des juges et des avocats qui arbitrent ces causes judiciaires, il faut être capable de vulgariser l'information.»

Dans le merveilleux monde du sport, elle rencontre parfois plus créatif qu'elle : «Pour un résultat positif à un test de testostérone, le sprinter américain Dennis Mitchell avait blâmé la consommation de bière et des relations sexuelles avec sa femme!»

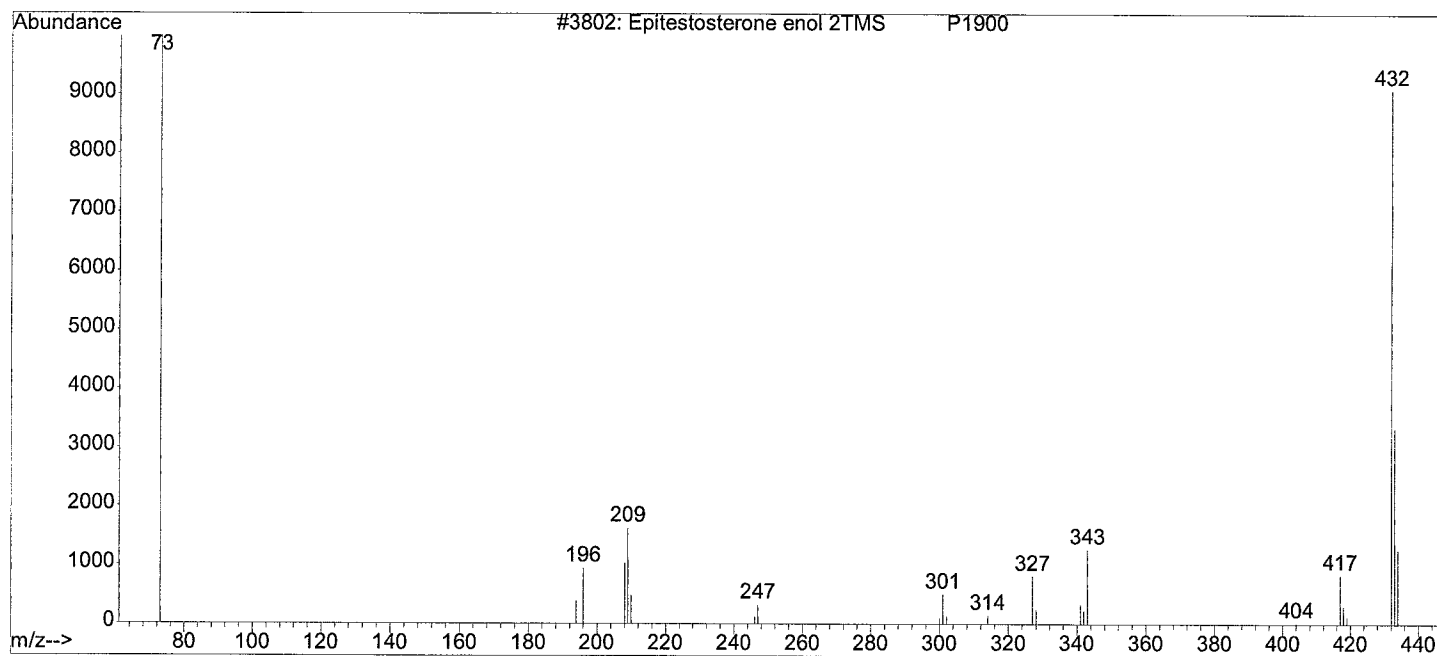
Epitestosterone enol 2TMS

P1900

Entry Number 3802 from C:\DATABASE\PMW_TOX2.L
CAS 000000-00-0
Melting Point 0
Boiling Point 0
Retention Index 2620
Mol Formula C₂₅H₄₄O₂Si₂
Mol Weight 432.287
Company ID Univ-Saar

Miscellaneous Information

Biomolecule %3802



No structure available for 000000-00-0

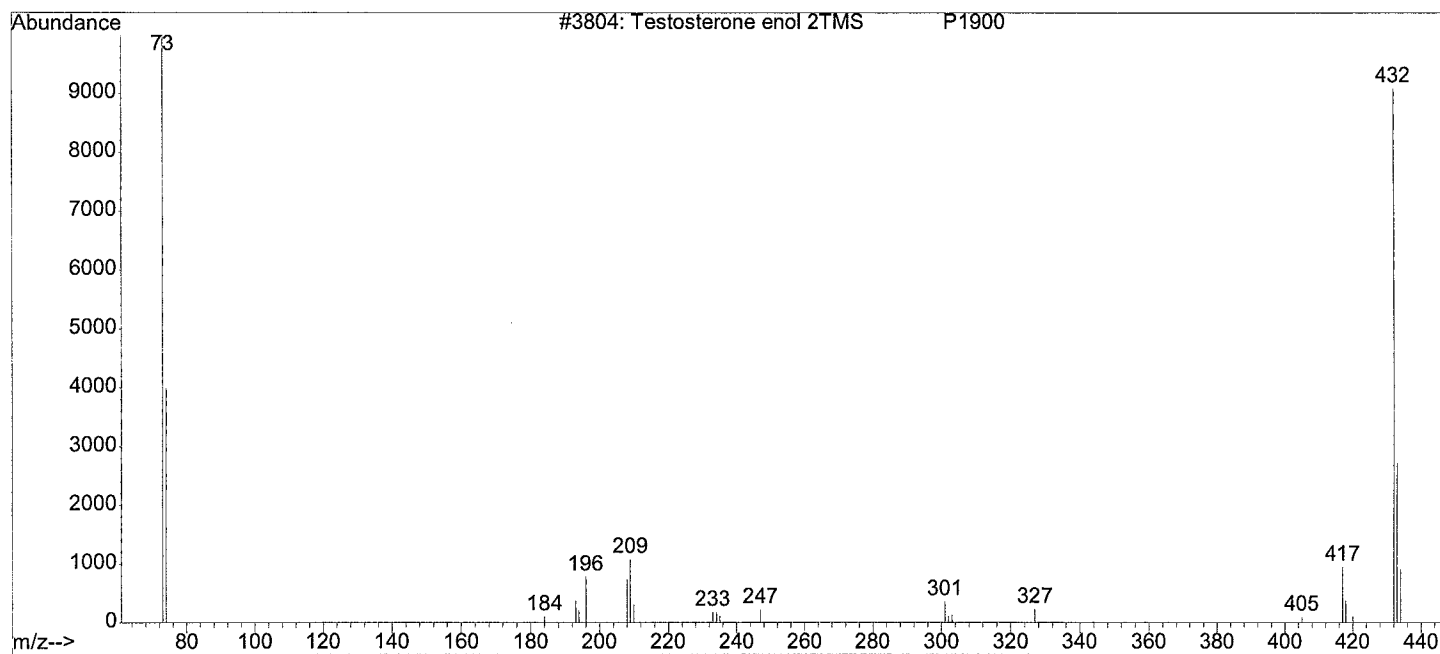
Testosterone enol 2TMS

P1900

Entry Number 3804 from C:\DATABASE\PMW_TOX2.L
CAS 000000-00-0
Melting Point 0
Boiling Point 0
Retention Index 2690
Mol Formula C₂₅H₄₄O₂Si₂
Mol Weight 432.287
Company ID Univ-Saar

Miscellaneous Information

Androgen %3804



No structure available for 000000-00-0

QUANTITATION REPORT FOR COC ON : GC-MS #2

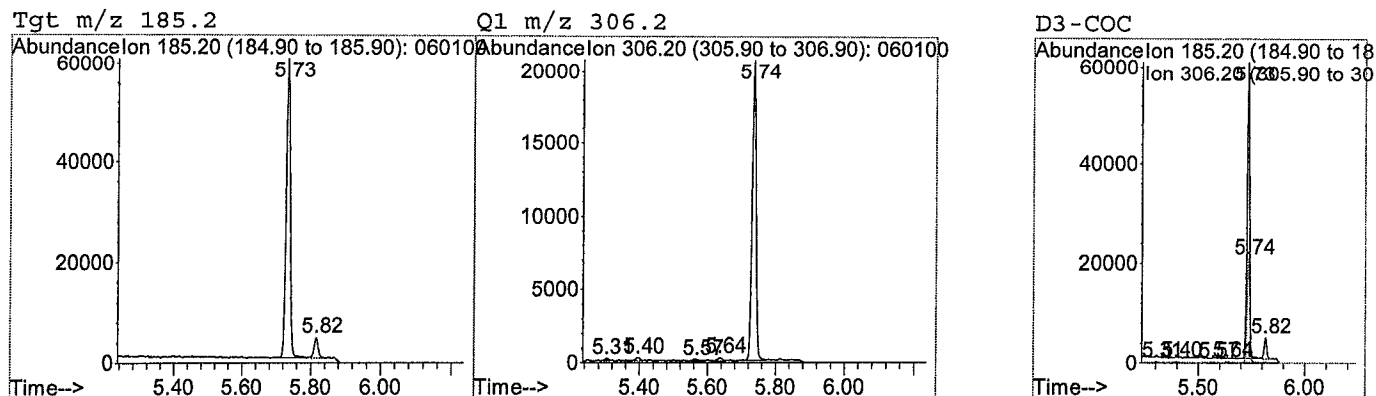
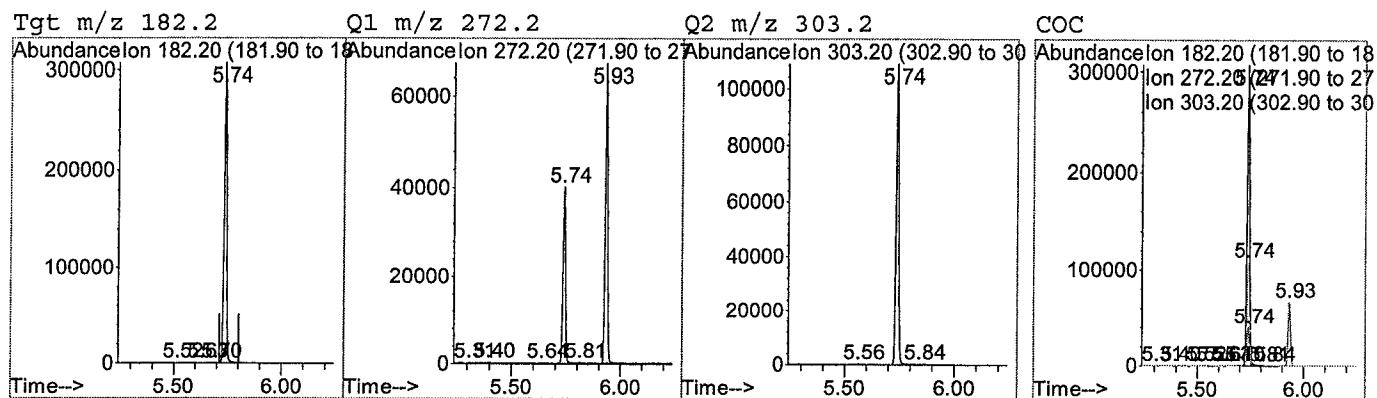
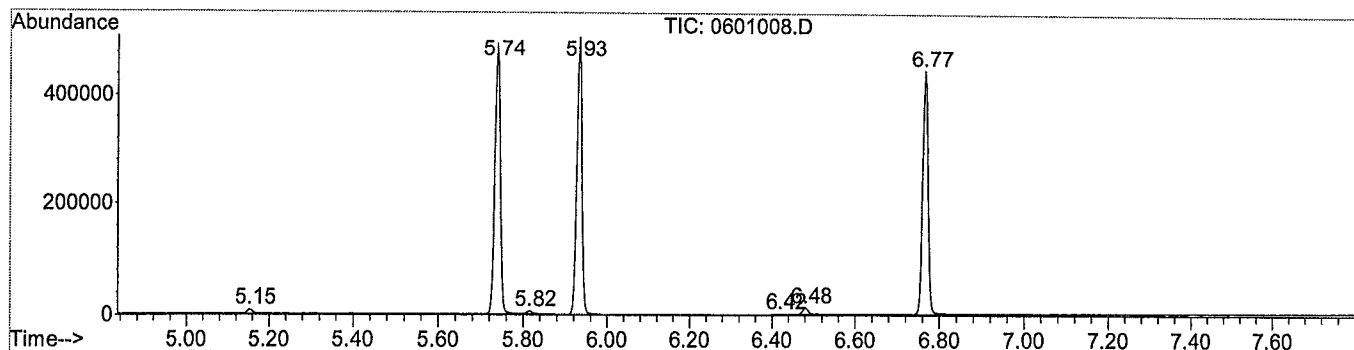
Data File : C:\HPCHEM\1\DATA\14MAY07\0601008.D
Tune File Name : C:\HPCHEM\1\5972\ATUNE.U
Tune Date : 14 May 2007 10:57 am Mult : 1
Acq Method Name : PMCO.C Calib date : 15 May 2007 11:43 am
Sample Name : STD 4: 500
Acquisition date : 14 May 2007 1:09 pm

Retention Time 5.74 COC +/- 1.00% = 5.68 - 5.80 min
Retention Time 5.73 D3-COC +/- 1.00% = 5.68 - 5.79 min
R.R.T. = 1.001 Unknown target ion / ISTD target ion = 5.17

COC => 182.2 = 254424 272.2 = 33074 303.2 = 88382
D3-COC => 185.2 = 49206 306.2 = 16696

COC => 272.2/182.2 = 13.0 +/- 20.0% rel = 10.2 - 15.4
COC => 303.2/182.2 = 34.7 +/- 20.0% rel = 27.5 - 41.3
D3-COC => 306.2/185.2 = 33.9 +/- 20.0% rel = 27.4 - 41.2

Concentration = 511.84 ** COC DETECTED **



COC : RT extraction window from 5.24 to 6.24 min
D3-COC : RT extraction window from 5.24 to 6.24 min

QUANTITATION REPORT FOR BE ON : GC-MS #2

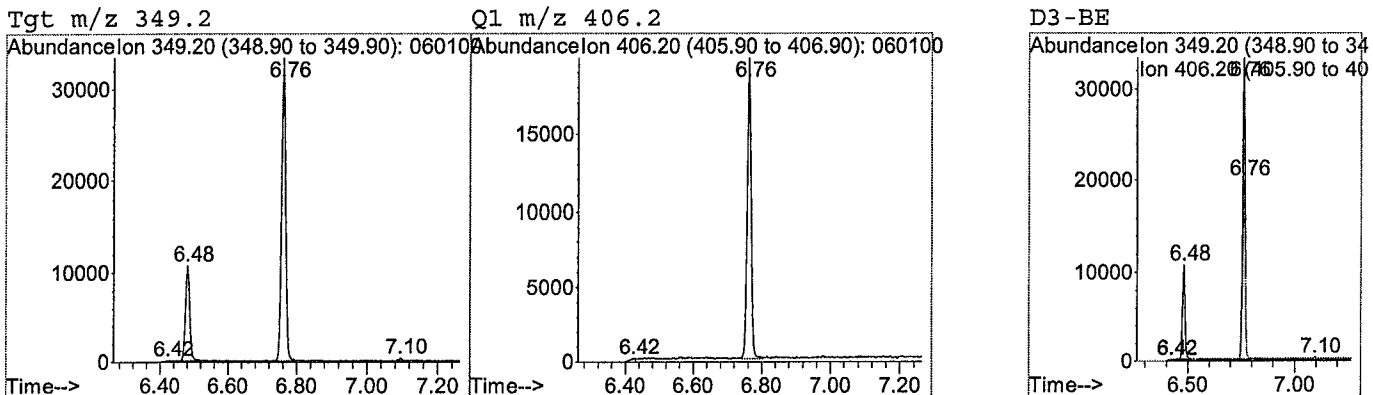
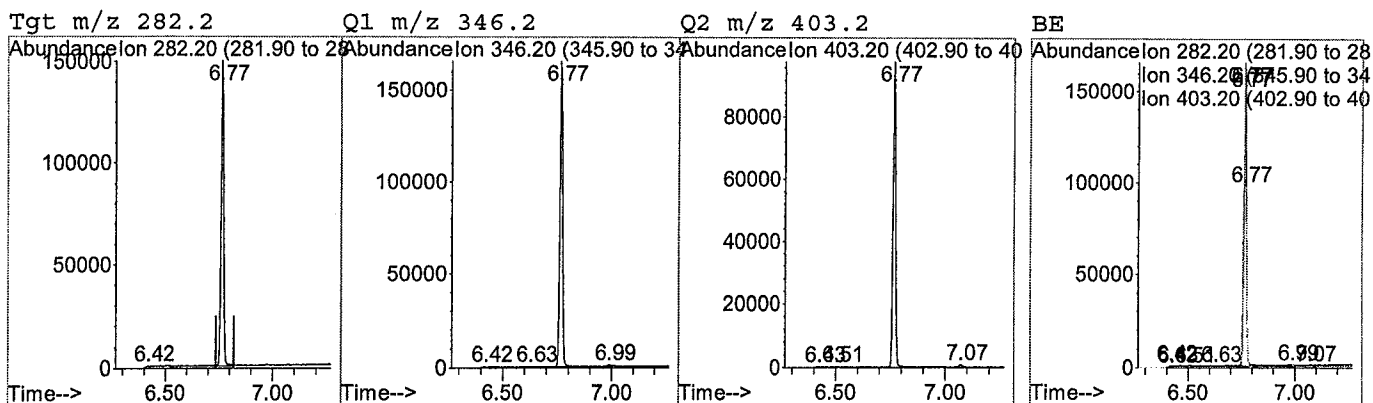
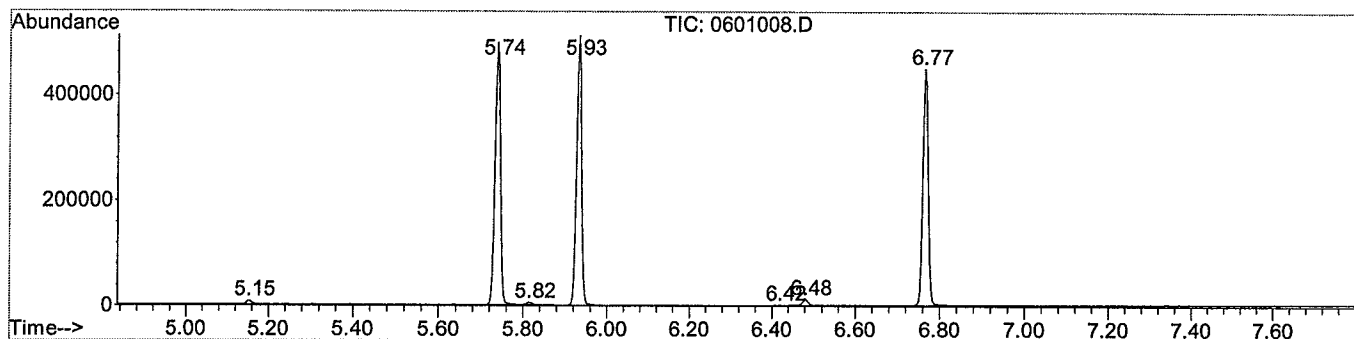
Data File : C:\HPCHEM\1\DATA\14MAY07\0601008.D
Tune File Name : C:\HPCHEM\1\5972\ATUNE.U
Tune Date : 14 May 2007 10:57 am Mult : 1
Acq Method Name : PMCOC.M Calib date : 15 May 2007 11:43 am
Sample Name : STD 4: 500
Acquisition date : 14 May 2007 1:09 pm

Retention Time 6.77 BE +/- 1.00% = 6.70 - 6.83 min
Retention Time 6.76 D3-BE +/- 1.00% = 6.70 - 6.83 min
R.R.T. = 1.001 Unknown target ion / ISTD target ion = 4.59

BE => 282.2 = 124757 346.2 = 140490 403.2 = 81378
D3-BE => 349.2 = 27166 406.2 = 15751

BE => 346.2/282.2 = 112.6 +/- 20.0% rel = 90.7 - 136.1
BE => 403.2/282.2 = 65.2 +/- 20.0% rel = 52.2 - 78.4
D3-BE => 406.2/349.2 = 58.0 +/- 20.0% rel = 44.8 - 67.2

Concentration = 514.17 ** BE DETECTED **



BE : RT extraction window from 6.27 to 7.27 min
D3-BE : RT extraction window from 6.26 to 7.26 min

QUANTITATION REPORT FOR CE ON : GC-MS #2

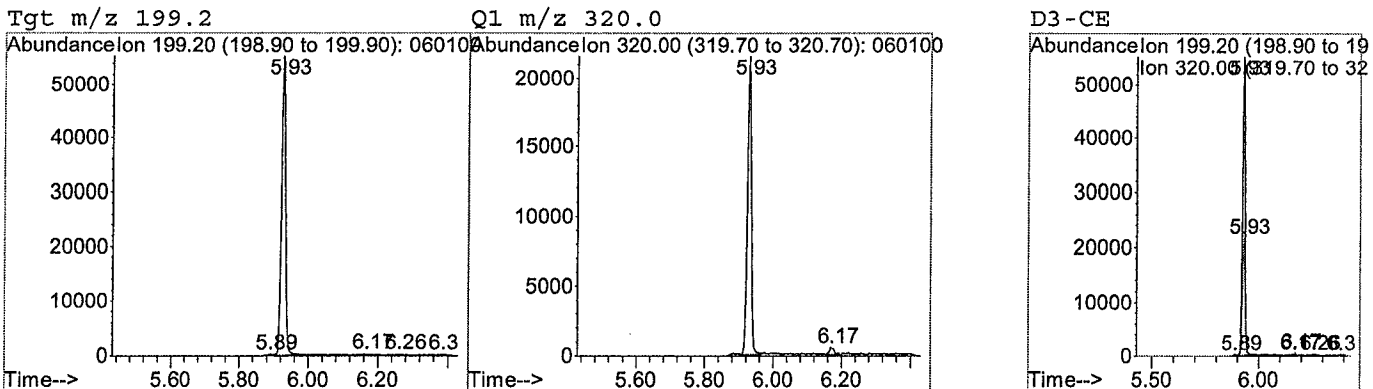
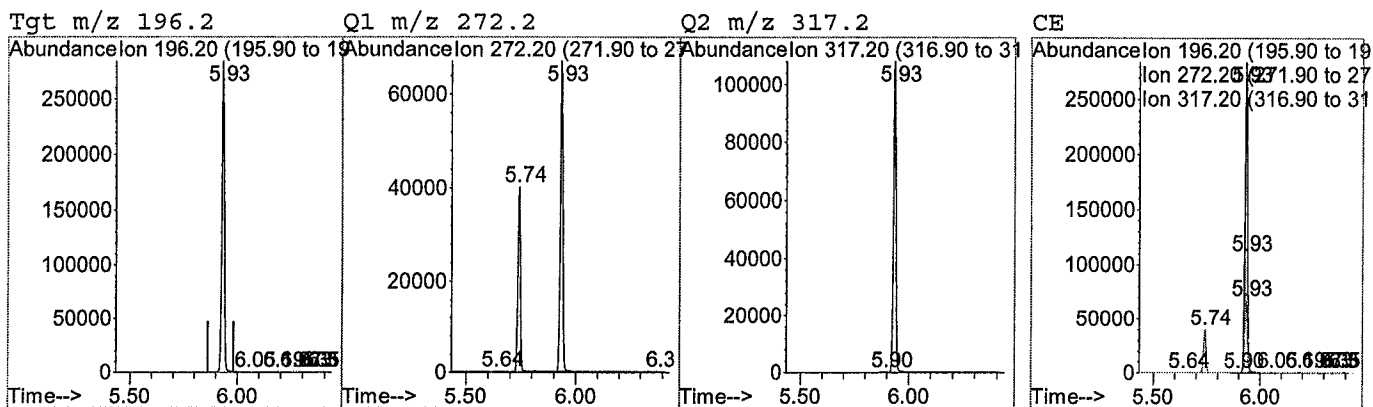
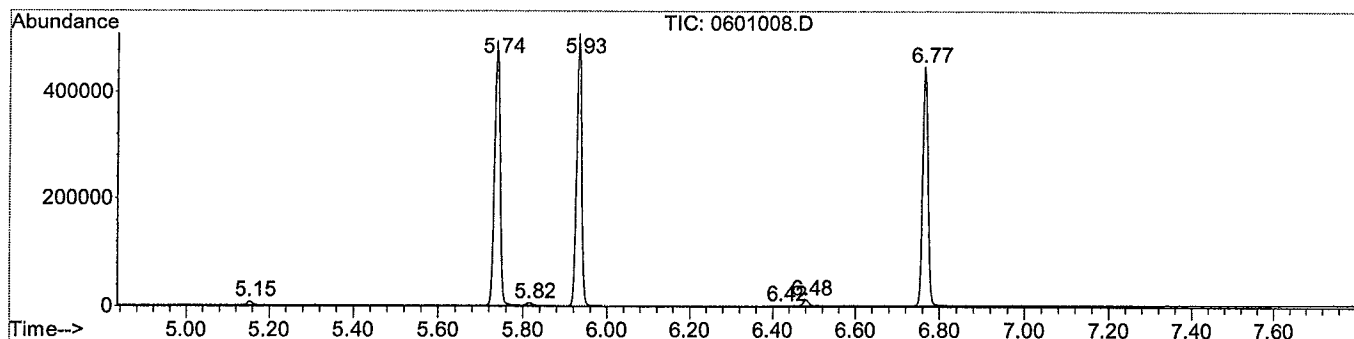
Data File : C:\HPCHEM\1\DATA\14MAY07\0601008.D
Tune File Name : C:\HPCHEM\1\5972\ATUNE.U
Tune Date : 14 May 2007 10:57 am Mult : 1
Acq Method Name : PMCO.C M Calib date : 15 May 2007 11:43 am
Sample Name : STD 4: 500
Acquisition date : 14 May 2007 1:09 pm

Retention Time 5.93 CE +/- 1.00% = 5.88 - 5.99 min
Retention Time 5.93 D3-CE +/- 1.00% = 5.87 - 5.99 min
R.R.T. = 1.001 Unknown target ion / ISTD target ion = 5.74

CE => 196.2 = 263481 272.2 = 53755 317.2 = 87306
D3-CE => 199.2 = 45884 320.0 = 17143

CE => 272.2/196.2 = 20.4 +/- 20.0% rel = 18.5 - 27.7
CE => 317.2/196.2 = 33.1 +/- 20.0% rel = 29.8 - 44.8
D3-CE => 320.0/199.2 = 37.4 +/- 20.0% rel = 29.4 - 44.2

Concentration = 595.28 ** CE DETECTED **

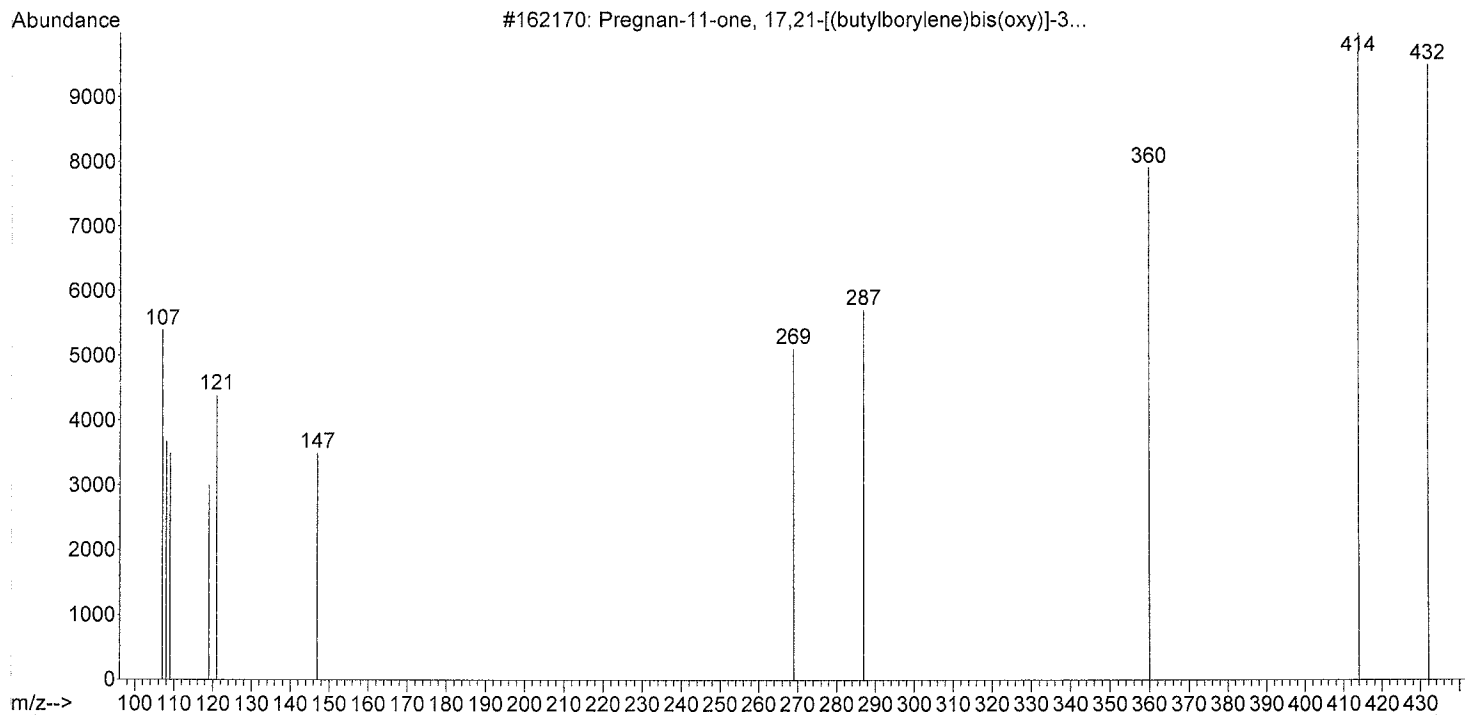


CE : RT extraction window from 5.43 to 6.43 min
D3-CE : RT extraction window from 5.43 to 6.43 min

Pregnan-11-one, 17,21-[(butylborylene)bis(oxy)]-3,20-dihydroxy-, (3.alpha.,5.beta.xy-, (3.alpha.,5.beta.,20S)-

Entry Number 162170 from C:\Database\NIST02.L
CAS 030888-51-8
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C₂₅H₄₁BO₅
Mol Weight 432.305
Company ID NIST 2002

Miscellaneous Information
NIST MS# 17500, Seq# M145004



No structure available for 030888-51-8

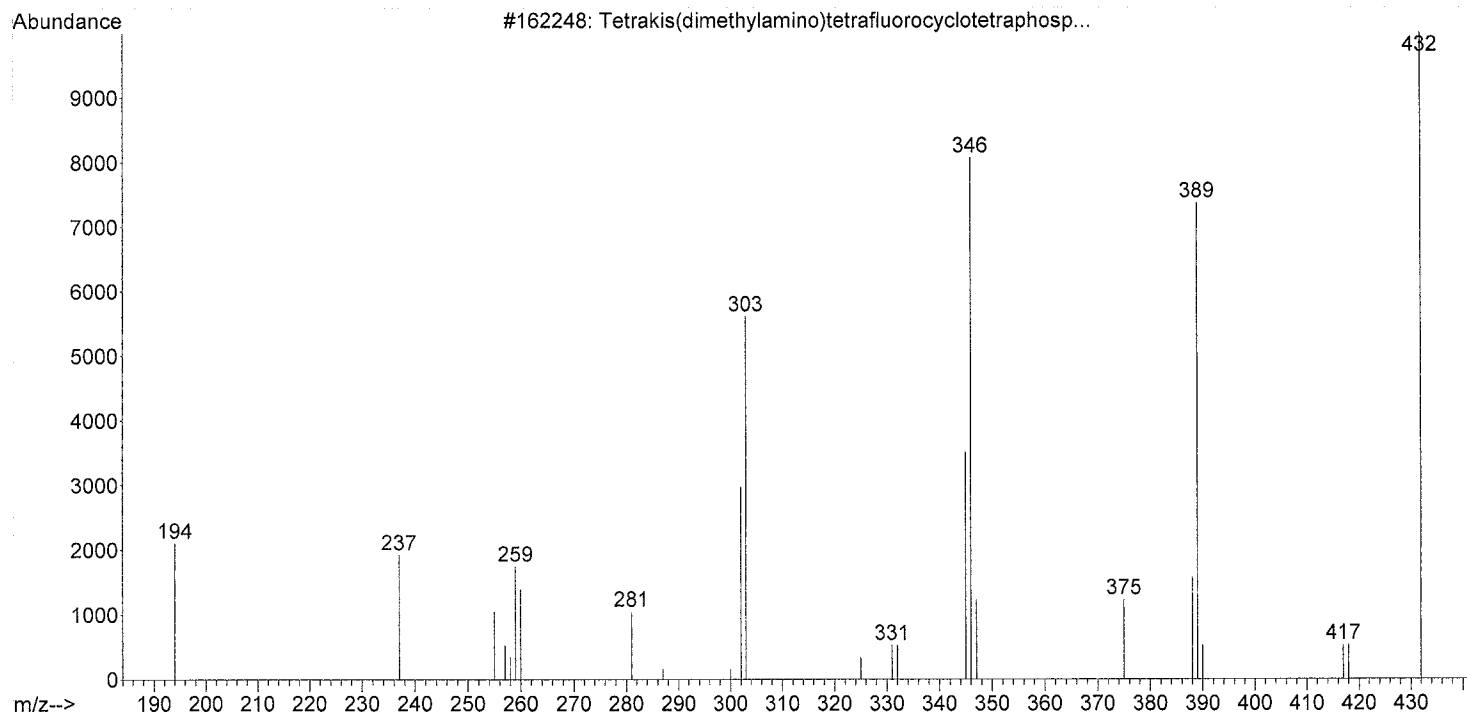
GDC01357.01

Tetrakis(dimethylamino)tetrafluorocyclotetraphosphazene

Entry Number 162248 from C:\Database\NIST02.L
CAS 1000137-65-2
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C₈H₂₄F₄N₈P₄
Mol Weight 432.101
Company ID NIST 2002

Miscellaneous Information

NIST MS# 137652, Seq# M145440, CAS number = 10⁹ + NIST MS#



No structure available for 1000137-65-2

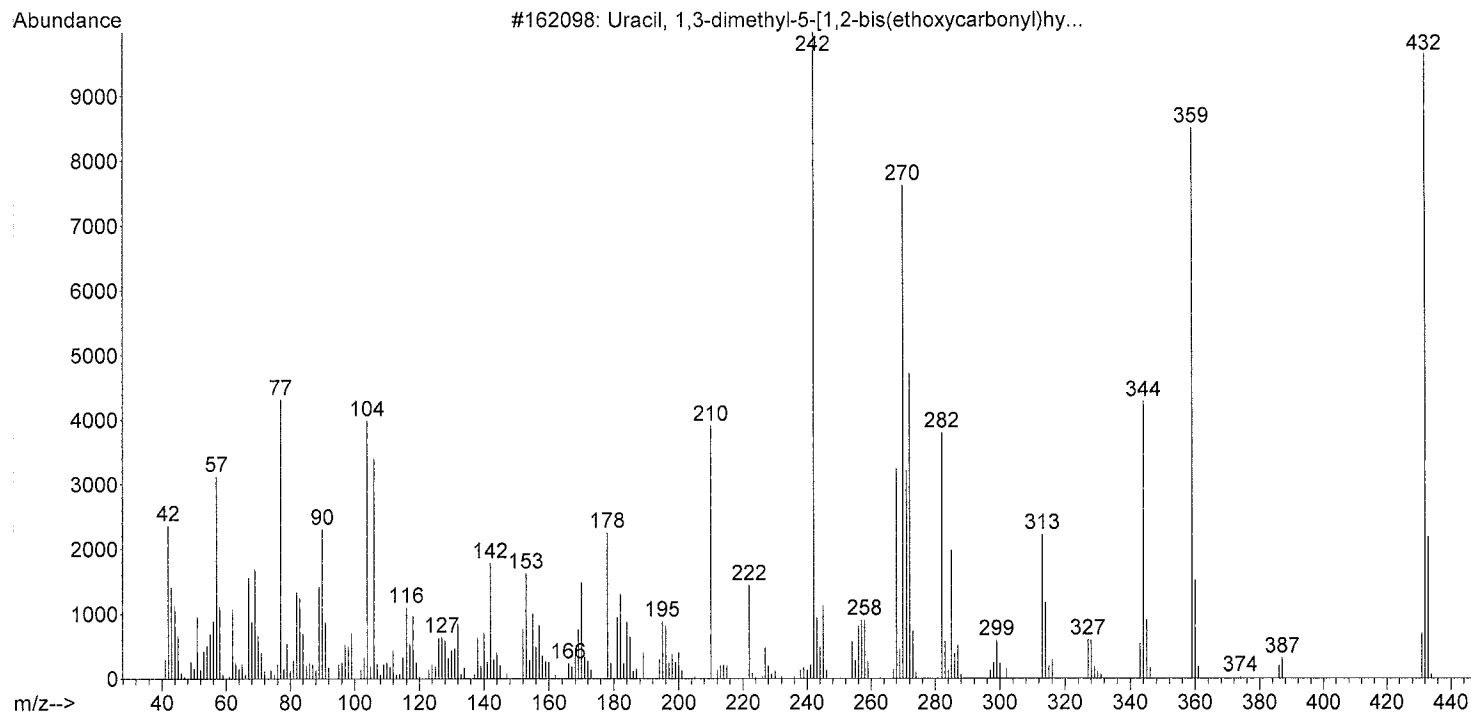
GDC01357.02

Uracil, 1,3-dimethyl-5-[1,2-bis(ethoxycarbonyl)hydrazino]-6-(2-benzylidenehydrazino)
(2-benzylidenehydrazino)-

Entry Number 162098 from C:\Database\NIST02.L
CAS 1000255-13-4
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C19H24N6O6
Mol Weight 432.176
Company ID NIST 2002

Miscellaneous Information

NIST MS# 255134, Seq# M124503, CAS number = 10⁹ + NIST MS#



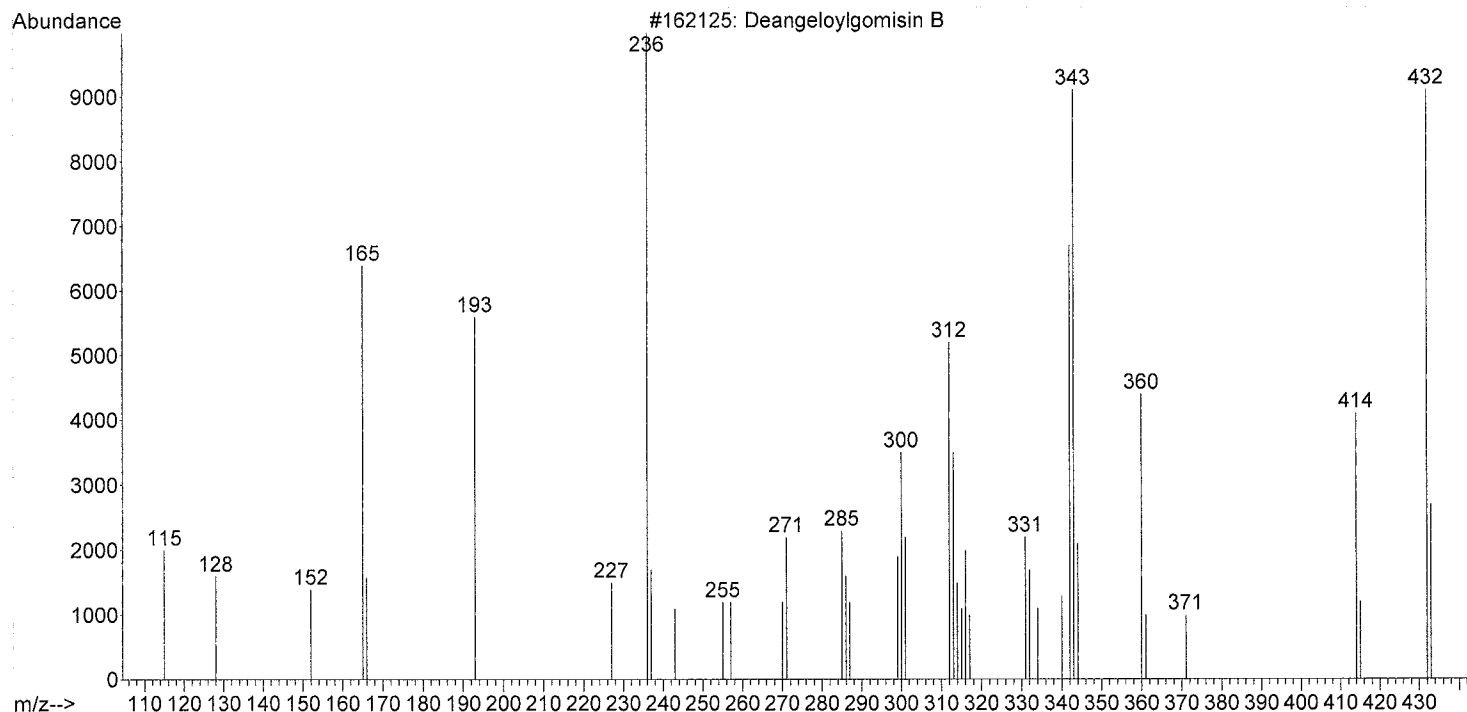
No structure available for 1000255-13-4

GDC01357.03

Deangeloylgomisin B

Entry Number 162125 from C:\Database\NIST02.L
CAS 058546-59-1
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C23H28O8
Mol Weight 432.178
Company ID NIST 2002

Miscellaneous Information
NIST MS# 100539, Seq# M122931



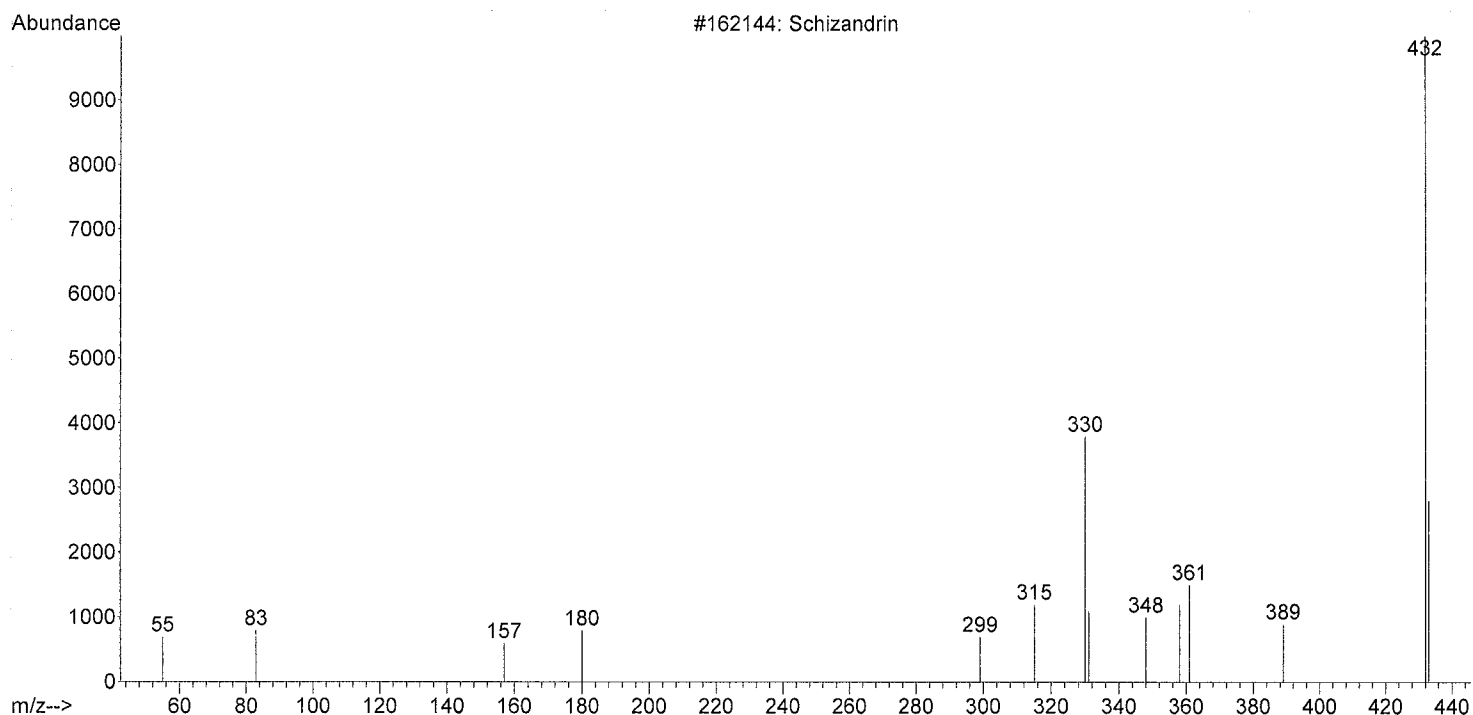
No structure available for 058546-59-1

GDC01357.04

Schizandrin

Entry Number 162144 from C:\Database\NIST02.L
CAS 007432-28-2
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C₂₄H₃₂O₇
Mol Weight 432.215
Company ID NIST 2002

Miscellaneous Information
NIST MS# 100535, Seq# M145438



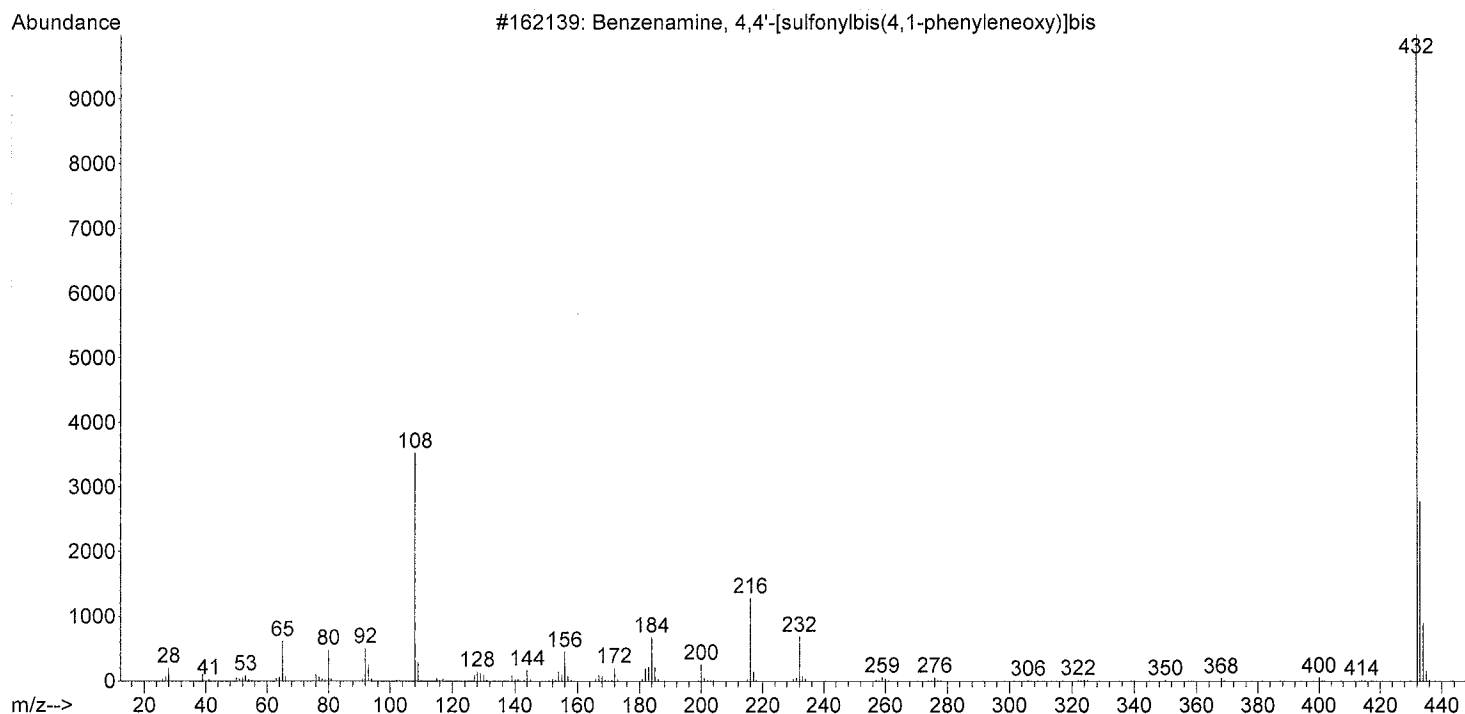
No structure available for 007432-28-2

GDC01357.05

Benzenamine, 4,4'-[sulfonylbis(4,1-phenyleneoxy)]bis-

Entry Number 162139 from C:\Database\NIST02.L
CAS 013080-89-2
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C24H20N2O4S
Mol Weight 432.114
Company ID NIST 2002

Miscellaneous Information
NIST MS# 237533, Seq# M145433



No structure available for 013080-89-2

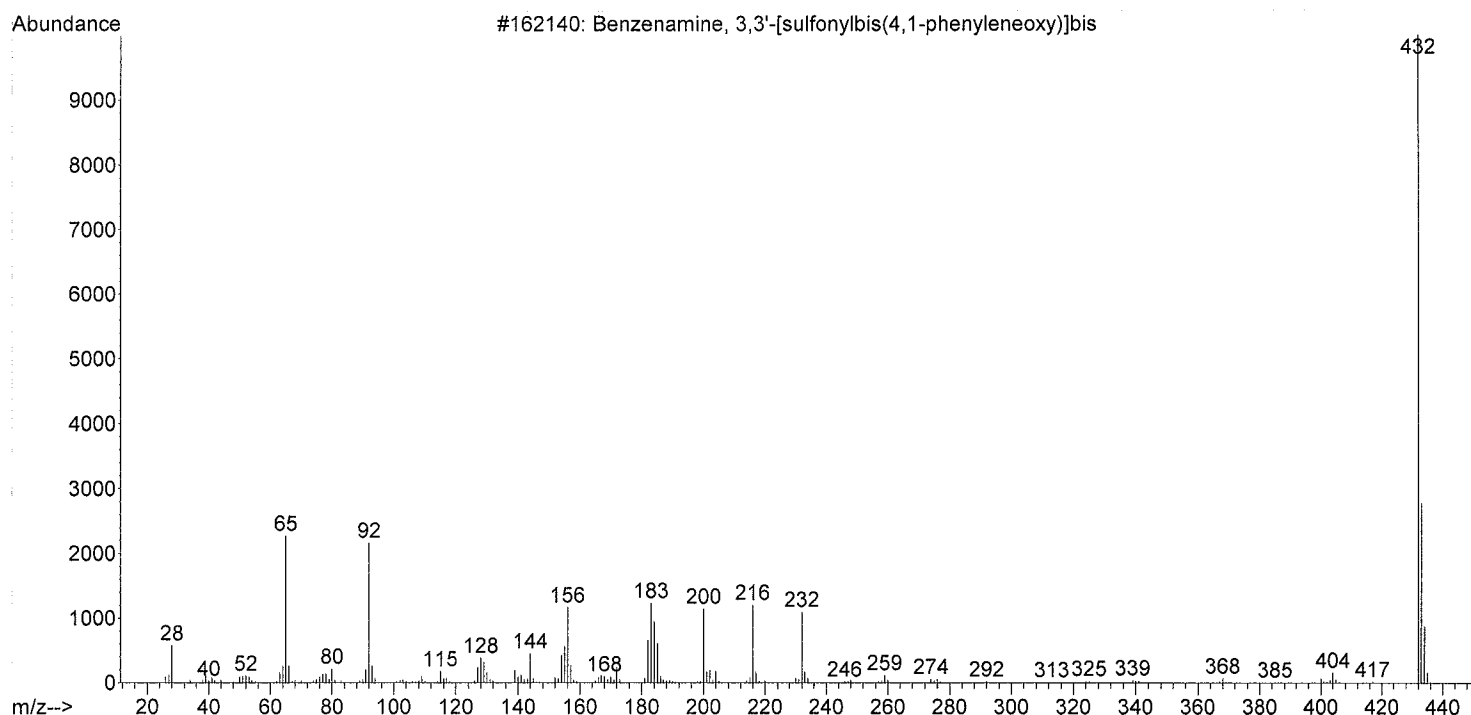
GDC01357.06

Benzenamine, 3,3'-[sulfonylbis(4,1-phenyleneoxy)]bis-

Entry Number 162140 from C:\Database\NIST02.L
CAS 030203-11-3
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C24H20N2O4S
Mol Weight 432.114
Company ID NIST 2002

Miscellaneous Information

NIST MS# 237532, Seq# M145445



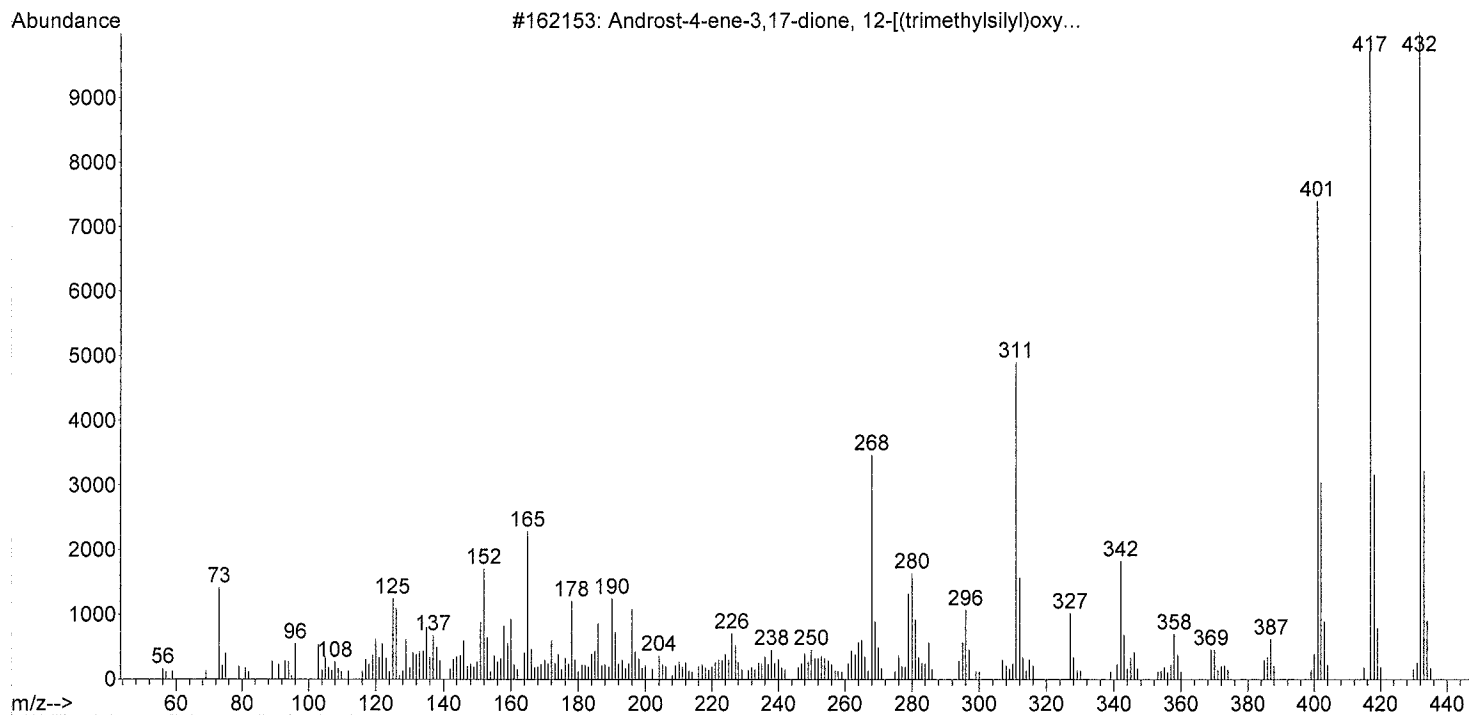
No structure available for 030203-11-3

GDC01357.07

Androst-4-ene-3,17-dione, 12-[(trimethylsilyl)oxy]-, bis(O-methyloxime), (12.beta. ethyloxime), (12.beta.)-

Entry Number 162153 from C:\Database\NIST02.L
CAS 069688-35-3
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C₂₄H₄₀N₂O₃Si
Mol Weight 432.281
Company ID NIST 2002

Miscellaneous Information
NIST MS# 54916, Seq# M145443



No structure available for 069688-35-3

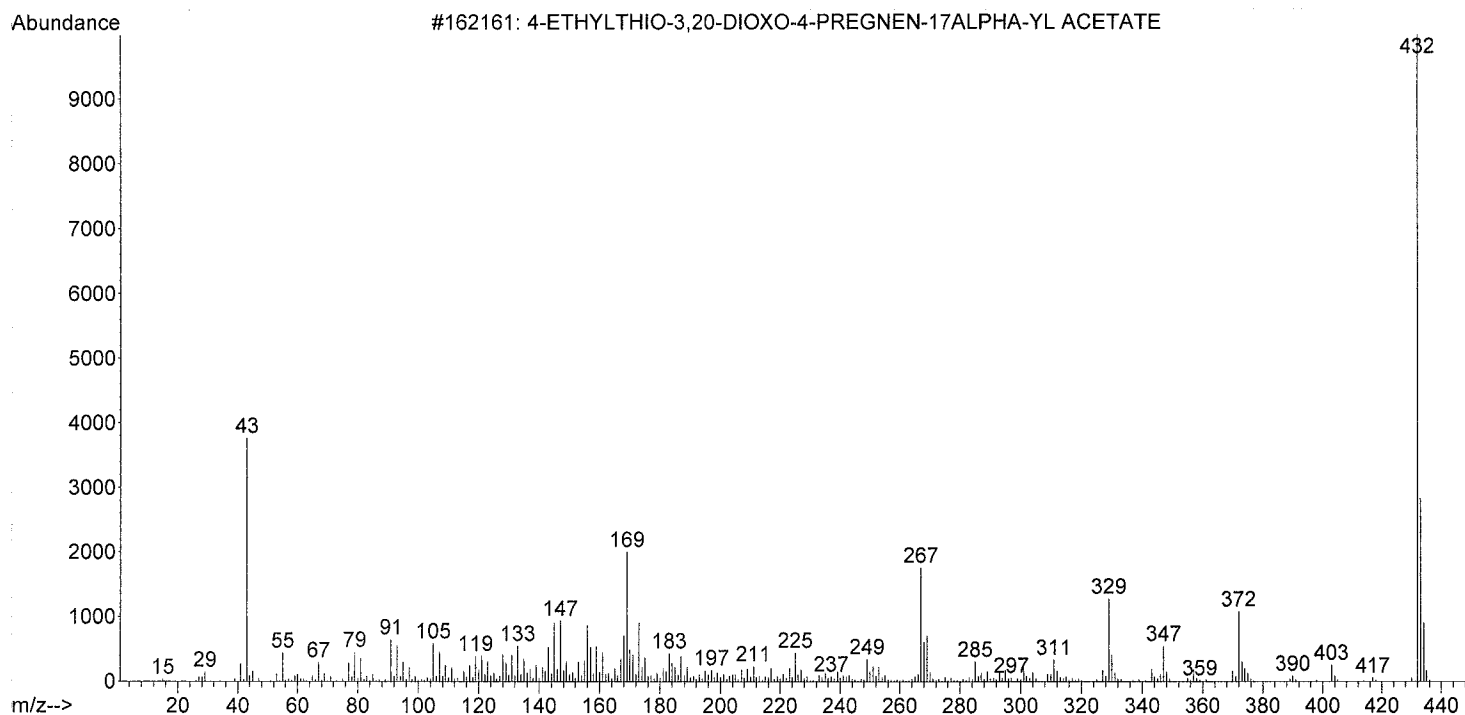
GDC01357.08

4-ETHYLTHIO-3,20-DIOXO-4-PREGNEN-17ALPHA-YL ACETATE

Entry Number 162161 from C:\Database\NIST02.L
CAS 1000244-57-0
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C25H36O4S
Mol Weight 432.233
Company ID NIST 2002

Miscellaneous Information

NIST MS# 244570, Seq# M145431, CAS number = 10⁹ + NIST MS#



No structure available for 1000244-57-0

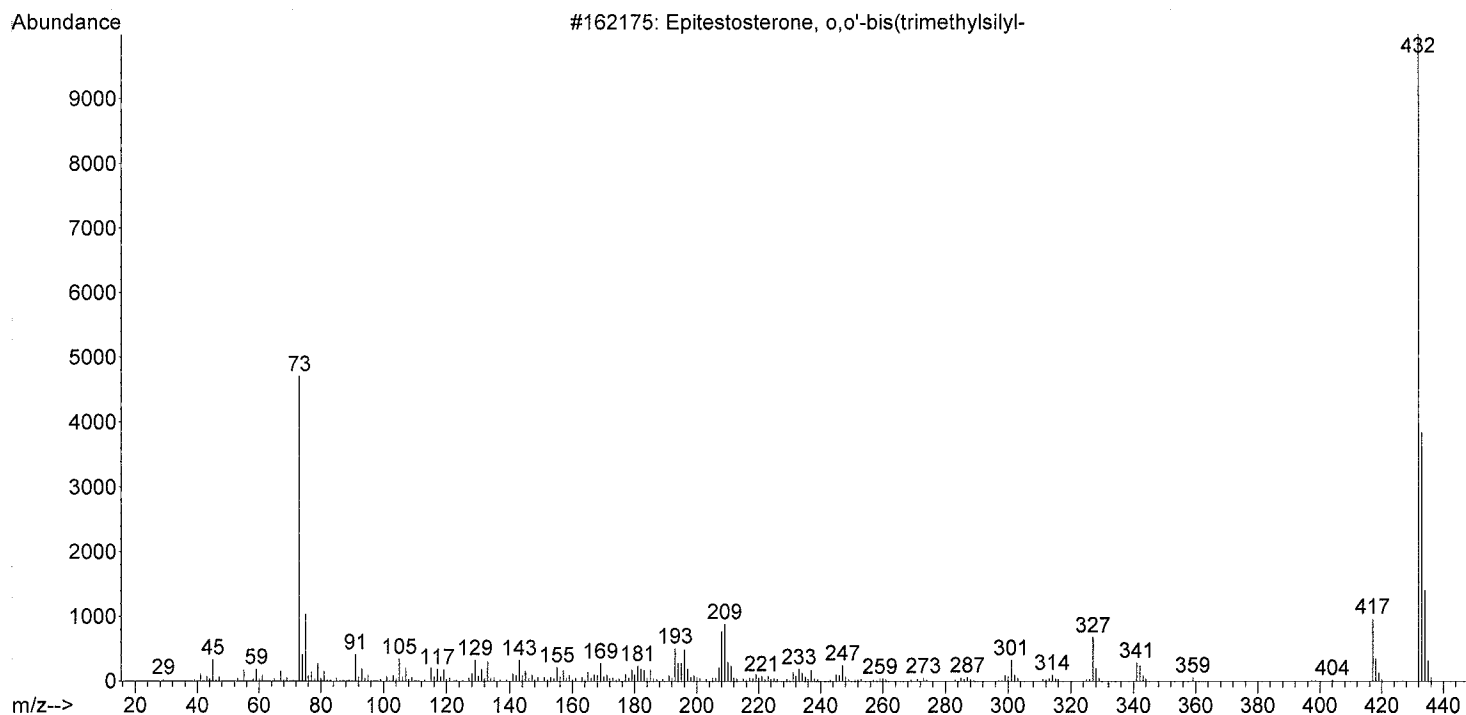
GDC01357.09

Epitestosterone, o,o'-bis(trimethylsilyl)-

Entry Number 162175 from C:\Database\NIST02.L
CAS 1000297-93-7
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C₂₅H₄₄O₂Si₂
Mol Weight 432.288
Company ID NIST 2002

Miscellaneous Information

NIST MS# 297937, Seq# M145432, CAS number = 10⁹ + NIST MS#



No structure available for 1000297-93-7

GDC01357.010

C43-A
Vol. 22 No. 22
Replaces C43-P
Vol. 20 No. 9

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

This document provides guidance on establishing uniform practices necessary to produce quality data for quantitation and identification of a drug or drug metabolite using the GC/MS method; specific quality assurance criteria for maintaining and documenting optional instrument performance are also presented. A guideline for global application developed through the NCCLS consensus process.



NCCLS...

Serving the World's Medical Science Community Through Voluntary Consensus

NCCLS is an international, interdisciplinary, nonprofit, standards-developing, and educational organization that promotes the development and use of voluntary consensus standards and guidelines within the healthcare community. It is recognized worldwide for the application of its unique consensus process in the development of standards and guidelines for patient testing and related healthcare issues. NCCLS is based on the principle that consensus is an effective and cost-effective way to improve patient testing and healthcare services.

In addition to developing and promoting the use of voluntary consensus standards and guidelines, NCCLS provides an open and unbiased forum to address critical issues affecting the quality of patient testing and health care.

PUBLICATIONS

An NCCLS document is published as a standard, guideline, or committee report.

Standard A document developed through the consensus process that clearly identifies specific, essential requirements for materials, methods, or practices for use in an unmodified form. A standard may, in addition, contain discretionary elements, which are clearly identified.

Guideline A document developed through the consensus process describing criteria for a general operating practice, procedure, or material for voluntary use. A guideline may be used as written or modified by the user to fit specific needs.

Report A document that has not been subjected to consensus review and is released by the Board of Directors.

CONSENSUS PROCESS

The NCCLS voluntary consensus process is a protocol establishing formal criteria for:

- the authorization of a project
- the development and open review of documents
- the revision of documents in response to comments by users
- the acceptance of a document as a consensus standard or guideline.

Most NCCLS documents are subject to two levels of consensus—"proposed" and "approved." Depending on

the need for field evaluation or data collection, documents may also be made available for review at an intermediate (i.e., "tentative") consensus level.

Proposed An NCCLS consensus document undergoes the first stage of review by the healthcare community as a proposed standard or guideline. The document should receive a wide and thorough technical review, including an overall review of its scope, approach, and utility, and a line-by-line review of its technical and editorial content.

Tentative A tentative standard or guideline is made available for review and comment only when a recommended method has a well-defined need for a field evaluation or when a recommended protocol requires that specific data be collected. It should be reviewed to ensure its utility.

Approved An approved standard or guideline has achieved consensus within the healthcare community. It should be reviewed to assess the utility of the final document, to ensure attainment of consensus (i.e., that comments on earlier versions have been satisfactorily addressed), and to identify the need for additional consensus documents.

NCCLS standards and guidelines represent a consensus opinion on good practices and reflect the substantial agreement by materially affected, competent, and interested parties obtained by following NCCLS's established consensus procedures. Provisions in NCCLS standards and guidelines may be more or less stringent than applicable regulations. Consequently, conformance to this voluntary consensus document does not relieve the user of responsibility for compliance with applicable regulations.

COMMENTS

The comments of users are essential to the consensus process. Anyone may submit a comment, and all comments are addressed, according to the consensus process, by the NCCLS committee that wrote the document. All comments, including those that result in a change to the document when published at the next consensus level and those that do not result in a change, are responded to by the committee in an appendix to the document. Readers are strongly encouraged to comment in any form and at any time on any NCCLS document. Address comments to the NCCLS Executive Offices, 940 West Valley Road, Suite 1400, Wayne, PA 19087, USA.

VOLUNTEER PARTICIPATION

Healthcare professionals in all specialties are urged to volunteer for participation in NCCLS projects. Please contact the NCCLS Executive Offices for additional information on committee participation.

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

Abstract

NCCLS document C43-A— *Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline* is intended to aid the laboratorian in developing appropriate procedures for the use of GC/MS in confirmation analyses. Its primary objective is to establish uniform practices necessary for producing quality data for quantitation and identification of a drug or drug metabolite. To support the scientific basis of the uniform practices, a brief overview of the techniques is provided. Specific quality assurance criteria for maintaining and documenting optimal instrument performance are presented.

NCCLS. *Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline*. NCCLS document C43-A (ISBN 1-56238-475-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002.

The NCCLS consensus process, which is the mechanism for moving a document through two or more levels of review by the healthcare community, is an ongoing process. Users should expect revised editions of any given document. Because rapid changes in technology may affect the procedures, methods, and protocols in a standard or guideline, users should replace outdated editions with the current editions of NCCLS documents. Current editions are listed in the *NCCLS Catalog*, which is distributed to member organizations, and to nonmembers on request. If your organization is not a member and would like to become one, and to request a copy of the *NCCLS Catalog*, contact the NCCLS Executive Offices. Telephone: 610.688.0100; Fax: 610.688.0700; E-Mail: exoffice@nccls.org; Website: www.nccls.org

C43-A

ISBN 1-56238-475-9

ISSN 0273-3099

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

Volume 22 Number 22

Larry D. Bowers, Ph.D., DABCC, Chairholder

David A. Armbruster, Ph.D., DABCC

Thomas Cairns, Ph.D., DABCC

Major John T. Cody, Ph.D., D-FTCB

Robert Fitzgerald, Ph.D., DABCC

Bruce A. Goldberger, Ph.D., DABFT

David Lewis

Leslie M. Shaw, Ph.D.



GDC01358.5

This publication is protected by copyright. No part of it may be reproduced, stored in a retrieval system, transmitted, or made available in any form or by any means (electronic, mechanical, photocopying, recording, or otherwise) without prior written permission from NCCLS, except as stated below.

NCCLS hereby grants permission to reproduce limited portions of this publication for use in laboratory procedure manuals at a single site, for interlibrary loan, or for use in educational programs provided that multiple copies of such reproduction shall include the following notice, be distributed without charge, and, in no event, contain more than 20% of the document's text.

Reproduced with permission, from NCCLS publication C43-A—*Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline* (ISBN 1-56238-475-9). Copies of the current edition may be obtained from NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

Permission to reproduce or otherwise use the text of this document to an extent that exceeds the exemptions granted here or under the Copyright Law must be obtained from NCCLS by written request. To request such permission, address inquiries to the Executive Director, NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

Copyright ©2002. The National Committee for Clinical Laboratory Standards.

Suggested Citation

(NCCLS. *Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline*. NCCLS document C43-A [ISBN 1-56238-475-9]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002.)

Proposed Guideline

April 2000

Approved Guideline

November 2002

ISBN 1-56238-475-9
ISSN 0273-3099

Committee Membership**Area Committee on Clinical Chemistry and Toxicology**

W. Gregory Miller, Ph.D.
Chairholder

Medical College of Virginia Hospital
Richmond, Virginia

Thomas L. Williams, M.D.
Vice-Chairholder

Methodist Hospital
Omaha, Nebraska

Subcommittee on GC/MS

Larry D. Bowers, Ph.D., DABCC
Chairholder

U.S. Anti-Doping Agency
Colorado Springs, Colorado

David A. Armbruster, Ph.D., DABCC

Abbott Laboratories
Abbott Park, Illinois

Thomas Cairns, Ph.D., D.Sc.

Psychomedics
Culver City, California

Major John T. Cody, Ph.D., D-FTCB

Academy of Health Sciences
Fort Sam Houston, Texas

Robert L. Fitzgerald, Ph.D., DABCC

VA (San Diego) Medical Center/UCSD
San Diego, California

Bruce A. Goldberger, Ph.D., DABFT

University of Florida College of Medicine
Gainesville, Florida

David Lewis

Signature Science
Austin, Texas

Leslie M. Shaw, Ph.D.

University of Pennsylvania
Philadelphia, Pennsylvania

Advisors

Bob Brittain

Varian Associates
Walnut Creek, California

Siu C. Chan, Ph.D.

University of Calgary
Calgary, Alberta, Canada

Yeshpal S. Chauhan, Ph.D.

Scarsdale, New York

John J. Fenton, Ph.D.

West Chester University
West Chester, Pennsylvania

Benjamin Gerson, M.D.

University Services
Philadelphia, Pennsylvania

Advisors (Continued)

Lieutenant L. Dan Hoey, Ph.D.

U.S. Navy Drug Screening Lab
Great Lakes, Illinois

Michael Leher, Ph.D.

Long Island Jewish Medical Center
New Hyde Park, New York

Joerg Norbert Pirl, Ph.D., DABFT

Illinois Department of Public Health
Chicago, Illinois

R. H. Barry Sample, Ph.D.

Quest Diagnostics
Atlanta, Georgia

Michael J. Welch, Ph.D.

National Institute of Standards & Technology
Gaithersburg, Maryland

Dr. Rafael de la Torre

Institute Municipal d'Investigacio Medica
Barcelona, Spain

Jennifer K. McGeary, M.T.(ASCP), M.S.H.A.
Staff Liaison

NCCLS
Wayne, Pennsylvania

Patrice E. Polgar
Editor/Project Manager

NCCLS
Wayne, Pennsylvania

Donna M. Wilhelm
Assistant Editor

NCCLS
Wayne, Pennsylvania

Active Membership (as of 1 October 2002)

Sustaining Members

Abbott Laboratories
American Association for
Clinical Chemistry
Beckman Coulter, Inc.
BD and Company
bioMérieux, Inc.
CLMA
College of American Pathologists
GlaxoSmithKline
Nippon Becton Dickinson Co., Ltd.
Ortho-Clinical Diagnostics, Inc.
Pfizer Inc
Roche Diagnostics, Inc.

Professional Members

AISAR-Associazione Italiana per lo
Studio degli
American Academy of Family
Physicians
American Association for
Clinical Chemistry
American Association for
Respiratory Care
American Chemical Society
American Medical Technologists
American Public Health Association
American Society for Clinical
Laboratory Science
American Society of Hematology
American Society for Microbiology
American Type Culture
Collection, Inc.
Asociacion Mexicana de
Bioquímica Clínica A.C.
Assn. of Public Health Laboratories
Assoc. Micro. Clinici Italiani-
A.M.C.L.I.
British Society for Antimicrobial
Chemotherapy
CADIME-Camara De Instituciones
De Diagnostico Medico
Canadian Society for Medical
Laboratory Science—Société
Canadienne de Science de
Laboratoire Médical
Clinical Laboratory Management
Association
COLA
College of American Pathologists
College of Medical Laboratory
Technologists of Ontario

College of Physicians and
Surgeons of Saskatchewan
ESCMID
Fundación Bioquímica Argentina
International Association of Medical
Laboratory Technologists
International Council for
Standardization in Haematology
International Federation of
Clinical Chemistry
Italian Society of Clinical
Biochemistry and Clinical
Molecular Biology
Japan Society of Clinical Chemistry
Japanese Committee for Clinical
Laboratory Standards
Joint Commission on Accreditation
of Healthcare Organizations
National Academy of Clinical
Biochemistry
National Association of Testing
Authorities – Australia
National Society for
Histotechnology, Inc.
Ontario Medical Association
Quality Management Program-
Laboratory Service
RCPA Quality Assurance Programs
PTY Limited
Sociedade Brasileira de Analises
Clinicas
Sociedad Espanola de Bioquímica
Clínica y Patología Molecular
Taiwanese Committee for Clinical
Laboratory Standards (TCCLS)
Turkish Society of Microbiology

Government Members

Association of Public Health
Laboratories
Armed Forces Institute of Pathology
BC Centre for Disease Control
Centers for Disease Control and
Prevention
Centers for Medicare & Medicaid
Services/CLIA Program
Centers for Medicare & Medicaid
Services
Chinese Committee for Clinical
Laboratory Standards
Commonwealth of Pennsylvania
Bureau of Laboratories
Department of Veterans Affairs
Deutsches Institut für Normung
(DIN)

FDA Center for Devices and
Radiological Health
FDA Center for Veterinary
Medicine
FDA Division of Anti-Infective
Drug Products
Iowa State Hygienic Laboratory
Massachusetts Department of
Public Health Laboratories
National Center of Infectious
and Parasitic Diseases (Bulgaria)
National Health Laboratory Service
(South Africa)
National Institute of Standards
and Technology
New York State Department of
Health
Ohio Department of Health
Ontario Ministry of Health
Pennsylvania Dept. of Health
Saskatchewan Health-Provincial
Laboratory
Scientific Institute of Public Health;
Belgium Ministry of Social
Affairs, Public Health and the
Environment
Swedish Institute for Infectious
Disease Control
Thailand Department of Medical
Sciences

Industry Members

AB Biodisk
Abbott Laboratories
Abbott Laboratories, MediSense
Products
Acrometrix Corporation
Alifax S.P.A.
Ammirati Regulatory Consulting
Anaerobe Systems
Assessor
AstraZeneca
AstraZeneca R & D
Boston
Avant Immunotherapeutics, Inc.
Aventis
Axis-Shield POC AS
Bayer Corporation – Elkhart, IN
Bayer Corporation – Tarrytown, NY
Bayer Corporation – West Haven,
CT
Bayer Medical Ltd.
BD
BD Biosciences – San Jose, CA
BD Consumer Products

BD Diagnostic Systems
 BD Italia S.P.A.
 BD VACUTAINER Systems
 Beckman Coulter, Inc.
 Beckman Coulter, Inc. Primary Care Diagnostics
 Beckman Coulter K.K. (Japan)
 Bio-Development SRL
 Bio-Inova Life Sciences International
 Bio-Inova Life Sciences North America
 BioMedia Laboratories Sdn Bhd
 BioMérieux (NC)
 bioMérieux, Inc. (MO)
 Biometrology Consultants
 Bio-Rad Laboratories, Inc.
 Bio-Rad Laboratories, Inc. - France
 Biotest AG
 Blaine Healthcare Associates, Inc.
 Bristol-Myers Squibb Company
 Canadian External Quality Assessment Laboratory
 Capital Management Consulting, Inc.
 Carl Schaper
 Checkpoint Development Inc.
 Chiron Corporation
 ChromaVision Medical Systems, Inc.
 Chronolab Ag
 Clinical Design Group Inc.
 Clinical Laboratory Improvement Consultants
 Cognigen
 Community Medical Center (NJ)
 Control Lab (Brazil)
 Copan Diagnostics Inc.
 Cosmetic Ingredient Review
 Cubist Pharmaceuticals
 Dade Behring Inc. - Deerfield, IL
 Dade Behring Inc. - Glasgow, DE
 Dade Behring Inc. - Marburg, Germany
 Dade Behring Inc. - Sacramento, CA
 Dade Behring Inc. - San Jose, CA
 David G. Rhoads Associates, Inc.
 Diagnostics Consultancy
 Diagnostic Products Corporation
 Eiken Chemical Company, Ltd.
 Elan Pharmaceuticals
 Electa Lab s.r.l.
 Enterprise Analysis Corporation
 Essential Therapeutics, Inc.
 EXPERTech Associates, Inc.
 F. Hoffman-La Roche AG
 Fort Dodge Animal Health
 General Hospital Vienna (Austria)

Gen-Probe
 GlaxoSmithKline
 Greiner Bio-One Inc.
 Helena Laboratories
 Home Diagnostics, Inc.
 IGEN Inc.
 Immunicon Corporation
 Instrumentation Laboratory
 International Technidyne Corporation
 IntraBiotics Pharmaceuticals, Inc.
 I-STAT Corporation
 Johnson and Johnson Pharmaceutical Research and Development, L.L.C.
 Kendall Sherwood-Davis & Geck
 LAB-Interlink, Inc.
 Laboratory Specialists, Inc.
 Labtest Diagnostica S.A.
 LifeScan, Inc. (a Johnson & Johnson Company)
 Lilly Research Laboratories
 Macemon Consultants
 Medical Device Consultants, Inc.
 Merck & Company, Inc.
 Minigrip/Zip-Pak
 Molecular Diagnostics, Inc.
 mvi Sciences (MA)
 Nabi
 Nichols Institute Diagnostics (Div. of Quest Diagnostics, Inc.)
 NimbleGen Systems, Inc.
 Nissui Pharmaceutical Co., Ltd.
 Nippon Becton Dickinson Co., Ltd.
 Norfolk Associates, Inc.
 Novartis Pharmaceuticals Corporation
 Ortho-Clinical Diagnostics, Inc. (Raritan, NJ)
 Ortho-Clinical Diagnostics, Inc. (Rochester, NY)
 Oxoid Inc.
 Paratek Pharmaceuticals
 Pfizer Inc
 Pharmacia Corporation
 Philips Medical Systems
 Powers Consulting Services
 Premier Inc.
 Procter & Gamble Pharmaceuticals, Inc.
 The Product Development Group
 QSE Consulting
 Quintiles, Inc.
 Radiometer America, Inc.
 Radiometer Medical A/S
 Replidyne
 Roche Diagnostics GmbH
 Roche Diagnostics, Inc.

Roche Laboratories (Div. Hoffmann-La Roche Inc.)
 Sarstedt, Inc.
 SARL Laboratoire Carron (France)
 Schering Corporation
 Schleicher & Schuell, Inc.
 Second Opinion
 Showa Yakuhin Kako Company, Ltd.
 Streck Laboratories, Inc.
 SurroMed, Inc.
 Synermed Diagnostic Corp.
 Sysmex Corporation (Japan)
 Sysmex Corporation (Long Grove, IL)
 The Clinical Microbiology Institute
 The Toledo Hospital (OH)
 Theravance Inc.
 Transasia Engineers
 Trek Diagnostic Systems, Inc.
 Versicor, Inc.
 Vetoquinol S.A.
 Visible Genetics, Inc.
 Vysis, Inc.
 Wallac Oy
 Wyeth-Ayerst
 Xyletech Systems, Inc.
 YD Consultant
 YD Diagnostics (Seoul, Korea)

Trade Associations

AdvaMed
 Association of Medical Diagnostic Manufacturers
 Japan Association Clinical Reagents Ind. (Tokyo, Japan)
 Medical Industry Association of Australia

Associate Active Members

31st Medical Group/SGSL (APO, AE)
 67th CSH Wuerzburg, GE (NY)
 121st General Hospital (CA)
 Academisch Ziekenhuis-VUB (Belgium)
 Acadiana Medical Laboratories, LTD (LA)
 Adena Regional Medical Center (OH)
 Advocate Healthcare Lutheran General (IL)
 Akershus Central Hospital and AFA (Norway)
 Albemarle Hospital (NC)
 Allegheny General Hospital (PA)

- Allina Health System (MN)
 Alton Ochsner Medical Foundation (LA)
 Antwerp University Hospital (Belgium)
 Arkansas Department of Health
 ARUP at University Hospital (UT)
 Armed Forces Research Institute of Medical Science (APO, AP)
 Associated Regional & University Pathologists (UT)
 Aurora Consolidated Laboratories (WI)
 Azienda Ospedale Di Lecco (Italy)
 Bay Medical Center (MI)
 Baystate Medical Center (MA)
 Bbaguas Duzen Laboratories (Turkey)
 Bermuda Hospitals Board
 Bo Ali Hospital (Iran)
 Brooks Air Force Base (TX)
 Broward General Medical Center (FL)
 Cadham Provincial Laboratory
 Calgary Laboratory Services
 Carilion Consolidated Laboratory (VA)
 Cathay General Hospital (Taiwan)
 Central Peninsula General Hospital (AK)
 Central Texas Veterans Health Care System
 Centre Hospitalier Regional del la Citadelle (Belgium)
 Centro Diagnostico Italiano (Milano, Italy)
 Champlain Valley Physicians Hospital (NY)
 Chang Gung Memorial Hospital (Taiwan)
 Changi General Hospital (Singapore)
 The Charlotte Hungerford Hospital (CT)
 Children's Hospital (LA)
 Children's Hospital (NE)
 Children's Hospital & Clinics (MN)
 Children's Hospital Medical Center (Akron, OH)
 Children's Hospital of Philadelphia (PA)
 Children's Medical Center of Dallas (TX)
 Clarian Health-Methodist Hospital (IN)
 Clendo Lab (Puerto Rico)
 Clinical Laboratory Partners, LLC (CT)
 CLSI Laboratories (PA)
- Columbia Regional Hospital (MO)
 Commonwealth of Kentucky
 Community Hospital of Lancaster (PA)
 CompuNet Clinical Laboratories (OH)
 Cook County Hospital (IL)
 Cook Children's Medical Center (TX)
 Covance Central Laboratory Services (IN)
 Danish Veterinary Laboratory (Denmark)
 Danville Regional Medical Center (VA)
 Delaware Public Health Laboratory
 Department of Health & Community Services (New Brunswick, Canada)
 DesPeres Hospital (MO)
 DeTar Hospital (TX)
 Detroit Health Department (MI)
 Diagnosticos da América S/A (Brazil)
 Dr. Everett Chalmers Hospital (New Brunswick, Canada)
 Doctors Hospital (Bahamas)
 Duke University Medical Center (NC)
 Dwight David Eisenhower Army Med. Ctr. (GA)
 E.A. Conway Medical Center (LA)
 Eastern Maine Medical Center
 East Side Clinical Laboratory (RI)
 Eastern Health (Vic., Australia)
 Elyria Memorial Hospital (OH)
 Emory University Hospital (GA)
 Esoterix Center for Infectious Disease (TX)
 Fairview-University Medical Center (MN)
 Federal Medical Center (MN)
 Florida Hospital East Orlando
 Focus Technologies (CA)
 Foothills Hospital (Calgary, AB, Canada)
 Fresenius Medical Care/Spectra East (NJ)
 Fresno Community Hospital and Medical Center
 Frye Regional Medical Center (NC)
 Gambro BCT (CO)
 Geisinger Medical Center (PA)
 Grady Memorial Hospital (GA)
 Guthrie Clinic Laboratories (PA)
 Hahnemann University Hospital (PA)
 Harris Methodist Erath County (TX)
- Harris Methodist Fort Worth (TX)
 Hartford Hospital (CT)
 Headwaters Health Authority (Alberta, Canada)
 Health Network Lab (PA)
 Health Partners Laboratories (VA)
 Heartland Regional Medical Center (MO)
 Highlands Regional Medical Center (FL)
 Hoag Memorial Hospital Presbyterian (CA)
 Holmes Regional Medical Center (FL)
 Holzer Medical Center (OH)
 Hopital du Sacre-Coeur de Montreal (Montreal, Quebec, Canada)
 Hôpital Maisonneuve – Rosemont (Montreal, Canada)
 Hospital for Sick Children (Toronto, ON, Canada)
 Hospital Sousa Martins (Portugal)
 Hotel Dieu Hospital (Windsor, ON, Canada)
 Houston Medical Center (GA)
 Huddinge University Hospital (Sweden)
 Hurley Medical Center (MI)
 Indiana University
 Innova Fairfax Hospital (VA)
 Institute of Medical and Veterinary Science (Australia)
 International Health Management Associates, Inc. (IL)
 Jackson Memorial Hospital (FL)
 Jersey Shore Medical Center (NJ)
 John C. Lincoln Hospital (AZ)
 John F. Kennedy Medical Center (NJ)
 John Peter Smith Hospital (TX)
 Kadlec Medical Center (WA)
 Kaiser Permanente Medical Care (CA)
 Kaiser Permanente (MD)
 Kantonsspital (Switzerland)
 Keller Army Community Hospital (NY)
 Kenora-Rainy River Regional Laboratory Program (Ontario, Canada)
 Kern Medical Center (CA)
 Kimball Medical Center (NJ)
 King Faisal Specialist Hospital (Saudi Arabia)
 King Khalid National Guard Hospital (Saudi Arabia)
 King's Daughter Medical Center (KY)

- Klinični Center (Slovenia)
 Laboratories at Bonfils (CO)
 Laboratoire de Santé Publique du
 Quebec (Canada)
 Laboratório Fleury S/C Ltda.
 (Brazil)
 Laboratory Corporation of America
 (NJ)
 Laboratory Corporation of
 America (MO)
 LAC and USC Healthcare
 Network (CA)
 Lakeland Regional Medical Center
 (FL)
 Lancaster General Hospital (PA)
 Langley Air Force Base (VA)
 LeBonheur Children's
 Medical Center (TN)
 L'Hotel-Dieu de Quebec (Canada)
 Libero Istituto Univ. Campus
 BioMedico (Italy)
 Louisiana State University
 Medical Center
 Maccabi Medical Care and Health
 Fund (Israel)
 Malcolm Grow USAF Medical
 Center (MD)
 Martin Luther King/Drew Medical
 Center (CA)
 Massachusetts General Hospital
 (Microbiology Laboratory)
 MDS Metro Laboratory Services
 (Burnaby, BC, Canada)
 Medical College of Virginia
 Hospital
 Medicare/Medicaid Certification,
 State of North Carolina
 Memorial Hospital at Gulfport (MS)
 Memorial Medical Center (IL)
 Memorial Medical Center (LA)
 Jefferson Davis Hwy
 Memorial Medical Center (LA)
 Napoleon Avenue
 Mercy Medical Center (IA)
 Methodist Hospital (TX)
 Methodist Hospitals of Memphis
 (TN)
 MetroHealth Medical Center (OH)
 Michigan Department of
 Community Health
 Mississippi Baptist Medical Center
 Monte Tabor - Centro Italo -
 Brasileiro de Promocao (Brazil)
 Montreal Children's Hospital
 (Canada)
 Montreal General Hospital
 (Canada)
- MRL Pharmaceutical Services, Inc.
 (VA)
 Nassau County Medical Center
 (NY)
 National Institutes of Health (MD)
 Naval Hospital - Corpus Christi
 (TX)
 Naval Surface Warfare Center (IN)
 Nebraska Health System
 New Britain General Hospital (CT)
 New England Fertility Institute
 (CT)
 New Mexico VA Health Care
 System
 New York University Medical
 Center
 North Carolina State Laboratory of
 Public Health
 North Shore - Long Island Jewish
 Health System Laboratories (NY)
 North Shore University Hospital
 (NY)
 Northwestern Memorial Hospital
 (IL)
 O.L. Vrouwziekenhuis (Belgium)
 Ordre professionnel des
 technologists médicaux du
 Québec
 Ospedali Riuniti (Italy)
 The Ottawa Hospital
 (Ottawa, ON, Canada)
 Our Lady of Lourdes Hospital (NJ)
 Our Lady of the Resurrection
 Medical Center (IL)
 Pathology and Cytology
 Laboratories, Inc. (KY)
 Pathology Associates Medical
 Laboratories (WA)
 The Permanente Medical Group
 (CA)
 Piedmont Hospital (GA)
 Pikeville Methodist Hospital (KY)
 Pocono Hospital (PA)
 Presbyterian Hospital of Dallas
 (TX)
 Providence Health Care
 Queen Elizabeth Hospital (Prince
 Edward Island, Canada)
 Queensland Health Pathology
 Services (Australia)
 Quest Diagnostics Incorporated
 (CA)
 Quintiles Laboratories, Ltd. (GA)
 Regions Hospital
 Reid Hospital & Health Care
 Services (IN)
 Research Medical Center (MO)
 Rex Healthcare (NC)
- Rhode Island Department of Health
 Laboratories
 Riyadh Armed Forces Hospital
 (Saudi Arabia)
 Royal Columbian Hospital (New
 Westminster, BC, Canada)
 Sacred Heart Hospital (MD)
 Saint Mary's Regional Medical
 Center (NV)
 St. Alexius Medical Center (ND)
 St. Anthony Hospital (CO)
 St. Anthony's Hospital (FL)
 St. Barnabas Medical Center (NJ)
 St-Eustache Hospital (Quebec,
 Canada)
 St. Francis Medical Ctr. (CA)
 St. John Hospital and Medical
 Center (MI)
 St. John Regional Hospital (St.
 John, NB, Canada)
 St. Joseph Hospital (NE)
 St. Joseph's Hospital - Marshfield
 Clinic (WI)
 St. Joseph's Medical Center (NY)
 St. Joseph Mercy Hospital (MI)
 St. Jude Children's Research
 Hospital (TN)
 St. Luke's Regional Medical
 Center (IA)
 St. Mary of the Plains Hospital
 (TX)
 St. Mary's Hospital & Medical
 Center (CO)
 St. Vincent Medical Center (CA)
 Ste. Justine Hospital (Montreal, PQ,
 Canada)
 Salina Regional Health Center (KS)
 San Francisco General Hospital
 (CA)
 Santa Clara Valley Medical Center
 (CA)
 Seoul Nat'l University Hospital
 (Korea)
 Shanghai Center for the
 Clinical Laboratory (China)
 South Bend Medical Foundation
 (IN)
 Southwest Texas Methodist Hospital
 (TX)
 South Western Area Pathology
 Service (Australia)
 Southern Maine Medical Center
 Specialty Laboratories, Inc. (CA)
 Stanford Hospital and Clinics (CA)
 State of Washington Department of
 Health
 Stony Brook University Hospital
 (NY)

Stormont-Vail Regional Medical Center (KS)
 Sun Health-Boswell Hospital (AZ)
 Swedish Medical Center – Providence Campus (WA)
 Tampa General Hospital (FL)
 Temple University Hospital (PA)
 Tenet Odessa Regional Hospital (TX)
 The Toledo Hospital (OH)
 Touro Infirmary (LA)
 Trident Regional Medical Center (SC)
 Tripler Army Medical Center (HI)
 Truman Medical Center (MO)
 UCSF Medical Center (CA)
 UNC Hospitals (NC)
 University College Hospital (Galway, Ireland)
 University Hospital (Gent) (Belgium)
 University Hospitals of Cleveland (OH)
 The University Hospitals (OK)
 University of Alabama-Birmingham Hospital

University of Alberta Hospitals (Canada)
 University of Colorado Health Science Center
 University of Chicago Hospitals (IL)
 University of Illinois Medical Center
 University of the Ryukyus (Japan)
 University of Texas M.D. Anderson Cancer Center
 University of Virginia Medical Center
 University of Washington
 UZ-KUL Medical Center (Belgium)
 VA (Denver) Medical Center (CO)
 Virginia Department of Health
 VA (Hines) Medical Center
 VA (Kansas City) Medical Center (MO)
 VA (Western NY) Healthcare System
 VA (San Diego) Medical Center (CA)
 VA (Tuskegee) Medical Center (AL)

VA Outpatient Clinic (OH)
 Vejle Hospital (Denmark)
 Washington Adventist Hospital (MD)
 Washoe Medical Center Laboratory (NV)
 West Jefferson Medical Center (LA)
 West Shore Medical Center (MI)
 Wilford Hall Medical Center (TX)
 William Beaumont Army Medical Center (TX)
 William Beaumont Hospital (MI)
 William Osler Health Centre (Brampton, ON, Canada)
 Williamsburg Community Hospital (VA)
 Winn Army Community Hospital (GA)
 Winnipeg Regional Health Authority (Winnipeg, Canada)
 Wishard Memorial Hospital (IN)
 Yonsei University College of Medicine (Korea)
 York Hospital (PA)

OFFICERS

Donna M. Meyer, Ph.D.,
 President
 CHRISTUS Health

Thomas L. Hearn, Ph.D.,
 President Elect
 Centers for Disease Control and Prevention

Emil Voelkert, Ph.D.,
 Secretary
 Roche Diagnostics GmbH

Gerald A. Hoeltge, M.D.,
 Treasurer
 The Cleveland Clinic Foundation

F. Alan Andersen, Ph.D.,
 Immediate Past President
 Cosmetic Ingredient Review

John V. Bergen, Ph.D.,
 Executive Director

Susan Blonshine, RRT, RPFT,
 FAARC
 TechEd

Wayne Brinster
 BD

Kurt H. Davis, FCSMLS, CAE
 Canadian Society for Medical Laboratory Science

Lillian J. Gill, M.S.
 FDA Center for Devices and Radiological Health

Robert L. Habig, Ph.D.
 Habig Consulting Group

Carolyn D. Jones, J.D., M.P.H.
 AdvaMed

BOARD OF DIRECTORS

Tadashi Kawai, M.D., Ph.D.
 International Clinical Pathology Center

J. Stephen Kroger, M.D., MACP
 COLA

Willie E. May, Ph.D.
 National Institute of Standards and Technology

Gary L. Myers, Ph.D.
 Centers for Disease Control and Prevention

Barbara G. Painter, Ph.D.
 Pharma Micro Consultancy, LLC

Judith A. Yost, M.A., M.T.(ASCP)
 Centers for Medicare & Medicaid Services

Contents

| | |
|---|-----|
| Abstract..... | i |
| Committee Membership..... | v |
| Active Membership..... | vii |
| Foreword..... | xv |
| The Quality System Approach..... | xvi |
| 1 Introduction..... | 1 |
| 2 Scope..... | 1 |
| 3 Standard Precautions..... | 1 |
| 4 Principles of GC/MS..... | 2 |
| 4.1 Gas Chromatography..... | 2 |
| 4.2 Interface..... | 3 |
| 4.3 Mass Spectrometry..... | 3 |
| 4.4 Data Acquisition..... | 7 |
| 5 Definitions..... | 10 |
| 6 Method Validation..... | 12 |
| 6.1 Specificity..... | 12 |
| 6.2 Trueness..... | 13 |
| 6.3 Precision (Repeatability and Reproducibility)..... | 14 |
| 6.4 Linearity..... | 14 |
| 6.5 Limit of Detection..... | 15 |
| 6.6 Limit of Quantitation..... | 16 |
| 6.7 Robustness..... | 16 |
| 7 Routine Instrument and Method Performance Verification..... | 16 |
| 7.1 Gas Chromatograph..... | 16 |
| 7.2 Mass Spectrometer..... | 17 |
| 7.3 Calibration..... | 18 |
| 7.4 Quality Control/Quality Assurance..... | 19 |
| 8 Identification Principles..... | 20 |
| 8.1 Gas Chromatograph..... | 20 |
| 8.2 Mass Spectral Identification..... | 21 |
| References..... | 24 |
| Additional References..... | 26 |
| Summary of Comments and Subcommittee Responses..... | 27 |
| Related NCCLS Publications..... | 33 |

Foreword

The detection of a drug in the biological fluid of an individual can have serious professional, financial, and social consequences. It is generally accepted that detection of a drug by a screening procedure must be confirmed by a second method based on a different analytical or physical principle. The purpose of the confirmation test is to decrease the probability of false-positives and to provide additional information and assurance about the identity of the detected compound.

Gas chromatography/mass spectrometry (GC/MS) is widely accepted in both scientific and legal arenas as one of the most powerful analytical techniques for the separation, quantification, and identification of drug analytes, especially at low concentrations. Technological advances have allowed introduction of bench-top GC/MS instrumentation into forensic and clinical toxicology laboratories. Further advances will continue to move state-of-the-art techniques such as gas- and liquid-phase chemical ionization, tandem mass spectrometry, high-resolution mass spectrometry, and high-performance liquid chromatography/mass spectrometry (HPLC/MS) into routine laboratory operation. Appropriate application of these analytical tools requires that the methods used are fit for their purpose and the instruments are operating correctly.

The Division of Workplace Programs, Substance Abuse and Mental Health Services Administration of the United States Department of Health and Human Services oversees the best-known drug testing program. The U.S. National Laboratory Certification Program has issued guidance documents for laboratories involved in the federal workplace drug-testing program. A similar program is under consideration in the European Union. Confirmatory assays are also used in clinical toxicology, forensic toxicology, and athletic drug testing. Currently available guidelines are not appropriate for all drug confirmation testing. The present guideline was developed to provide assistance in developing confirmation tests that are fit for the analytical purpose in each of these areas.

This guideline addresses the instrumental and methodological issues in developing a chromatographic - mass spectrometric method, routine performance of the analysis, and continued quality assurance. The chain of custody, while an important part of any test result to be submitted to the judicial system, is not discussed here. Guidelines for sample collection and screening testing have been published. Refer to the most current edition of NCCLS document T/DM8—*Urine Drug Testing in the Clinical Laboratory* for recommendations on sample collection and screening testing.

Key Words

Athletic drug testing, clinical toxicology, drugs of abuse, forensic toxicology, gas chromatography, magnetic sector mass spectrometer, mass spectrometry, quadrupole mass spectrometer, tandem mass spectrometry

A Note on Terminology

NCCLS, as a global leader in standardization and harmonization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. NCCLS recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in NCCLS, ISO, and CEN documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. In light of this, NCCLS recognizes that harmonization of terms facilitates the global application of standards and deserves immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

Of particular note in C43-A, are several terms whereby NCCLS intends to eliminate confusion over time, through its commitment to harmonization. For instance, the term "accuracy" comprises three different concepts that ISO documents capture with three distinct terms; i.e., "accuracy," "trueness," and "bias." Also in the context of this guideline, the term "precision" is defined the way ISO defines "uncertainty." To facilitate understanding, all ISO terms are defined in the guideline's "Definitions" section under the terms "accuracy" and "precision."

All terms and definitions will be reviewed for consistency with international use, and revised appropriately during the next scheduled revision of this guideline.

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care*. The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

QSEs

| | |
|------------------------|------------------------|
| Documents & Records | Information Management |
| Organization | Occurrence Management |
| Personnel | Assessment |
| Equipment | Process Improvement |
| Purchasing & Inventory | Service & Satisfaction |
| Process Control | Facilities & Safety |

C43-A addresses the following quality system essentials (QSEs):

| Documents & Records | Organization | Personnel | Equipment | Purchasing & Inventory | Process Control | Information Management | Occurrence Management | Assessment | Process Improvement | Service & Satisfaction | Facilities & Safety |
|---------------------|--------------|-----------|-----------|------------------------|-----------------|------------------------|-----------------------|------------|---------------------|------------------------|---------------------|
| | | | | | X | | | | | | |

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*.

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information. The arrow depicts the sequence, from left to right, that any clinical laboratory follows. In addition, the necessary steps or subprocesses are listed below them.

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

1 Introduction

Gas chromatography/Mass spectrometry (GC/MS) is generally accepted as the "gold standard" for identification and quantitation of drug analytes. As such, it is frequently used to confirm presumptive positive drug screening tests performed by immunoassay, thin-layer chromatography, high-performance liquid chromatography, or gas chromatography. The confidence in the ability of GC/MS to provide unequivocal analytical data is based on recognition of its reproducibility, repeatability, specificity, and trace detection capabilities. While this confidence is well founded, the measurement and identification of trace levels of compounds in complex biological matrices such as urine, hair, blood, bile, or organ tissue present a unique problem. Since GC/MS confirmation tests are applied in areas of clinical and forensic science other than drugs of abuse testing, it seems appropriate to establish broader criteria.

2 Scope

In drug analysis, GC/MS is used either to increase confidence in the identification of an unknown compound or to improve the limits of detection or quantitation through increased analytical specificity. Because of this unique combination of identification and quantitation capabilities, GC/MS methods, particularly confirmation methods, require a specific set of criteria for validation of methods and for performance verification in routine analysis.

There are two broad classes of drug analysis performed with GC/MS instrumentation. For some compounds, quantitative concentration thresholds have been established, on scientific and administrative grounds, to determine the presence of the drug or drug metabolite. When the threshold concentration, threshold ratio of amounts, or other defined parameter is exceeded, the compound is deemed to be present or to be present in nonphysiological amounts. In these cases, the performance of the method and instrument at the threshold has particular importance. The best-known example of the threshold approach was the development of specific administrative threshold concentrations and criteria for identification of five drugs of abuse for the federal drug-testing program.¹ For some other drugs or drug metabolites, however, detection at any documentable concentration is of concern. For these nonthreshold compounds, performance criteria for identification may be more important than the ability to quantify.

Although bench top GC/MS instrumentation has become more available and easier to use, a uniform practice must be established and maintained to provide acceptable evidence in an administrative appeal hearing or legal setting. Continuing improvements in theory and instrumentation will facilitate the use of new techniques, such as GC/MS/MS, in routine analysis. Thus, there is a need to define uniform practices not only for routine GC/MS methods, but also for the application of these more sophisticated approaches.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management

of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections*.

4 Principles of GC/MS

GC/MS is one of a number of hybrid techniques that couple two analytical techniques to achieve a synergistic improvement in analytical performance. The appropriate operation of each technique is necessary to achieve an analytical performance objective.

4.1 Gas Chromatography

GC separates molecules by differences in their equilibrium distribution between a gaseous mobile phase and a liquid or solid stationary phase. The degree of separation between the different components in a mixture is affected by the mobile phase, its flow rate, the stationary phase used, and the temperature and/or rate of temperature change. The emergence of the analyte from the column gives rise to a Gaussian-shaped chromatographic peak. The time that has elapsed between injection and the time when the chromatographic peak apex appears, corresponding to the elution of the maximum concentration of analyte, is called the "retention time." If an unknown compound has the same retention time as a reference material under the same chromatographic conditions, the result is consistent with the presumption, but does not prove, that the two compounds are the same.

4.1.1 Sample Introduction

Because the analyte must be in the gas phase for separation, the sample introduction system must transform the liquid sample into a gas. There are a variety of sample introduction techniques presently available in GC, including isothermal split or splitless, temperature-programmed split or splitless, direct, and on-column. Each technique has advantages and disadvantages. All techniques require the sample entry port to be sealed to isolate the mobile phase stream from the outside environment. This requires either a septum or an alternate sealing system that is repeatedly penetrated by a needle. Because most injection techniques require sufficient heat to rapidly vaporize both the injection solvent and the analyte(s), components dislodged and volatilized from the septum can cause problems. Small amounts (ng) of analyte are introduced into the column. Due to the relatively large surface area of the injection port, its design, the inertness of the materials used, and the cleanliness of those portions which come in contact with the sample are critical for efficient, reproducible sample introduction.

4.1.2 Columns

Two types of GC columns exist: packed and open tubular (capillary). Packed columns are very infrequently used in conjunction with mass spectrometers and will not be discussed. Capillary columns are small diameter (0.10 to 0.53 mm inside diameter) tubes of fused silica. The stationary phase is a cross-linked annulus on the inner wall of the tube. The stationary phase is a polysiloxane or polysilarylene polymer backbone on which functional groups such as methyl, phenyl, cyanopropyl, or trifluoroacetyl provide sites for interaction with the compounds to be separated. The amount and polarity of the stationary phase, the temperature of the column, and the mobile phase flow rate are the major determining factors for separation of two compounds.

With respect to GC/MS, the major impact of the column, other than the ability to separate the compounds of interest, is the continuous degradation and elution of the phase, called "column bleed." Since all compounds entering the mass spectrometer contribute to the final signal, column bleed contributes to system background. Column bleed can be minimized by operating the column within the manufacturer's temperature range limits, by excluding oxygen and other contaminants from the mobile phase, by introducing samples that do not degrade the phase, and by selecting polysilarylene-based bonded phases.

4.2 Interface

The interface provides continuous introduction of the gaseous chromatographic effluent into the mass spectrometer ion source. Jet separator, membrane separator, or effusion separator interfaces were used to enrich the ratio of sample to carrier gas. Wide-bore (megabore), open-tubular GC columns generally require an "open-split" interface in which a portion of the effluent is discarded without enrichment of the sample relative to the carrier gas. With the widespread use of narrow-bore capillary GC columns, all of the column effluent can be directed into the ion source using a direct interface. In general, the interface is relatively trouble-free, although careful temperature control of the transfer line between the gas chromatograph and mass spectrometer is required.

4.3 Mass Spectrometry

The ability of mass spectrometry to obtain information related to the structure of the compound complements the separation capabilities of GC. Molecules entering the mass spectrometer are ionized and may undergo fragmentation. The pattern of fragments and their relative amounts are characteristic of the chemical structure of a compound but may not be unique for that compound. A mass spectrum is the two-dimensional plot of the relative abundance versus the mass-to-charge ratio of the ions. When the abundances of all ions in the mass spectrum are summed and plotted as a function of elution time, the plot is called a "total ion chromatogram" (TIC). An ion chromatogram is the two-dimensional plot of the abundance of a particular mass-to-charge ratio (m/z) versus the retention time of a GC/MS run.

4.3.1 Ion Source

As the GC effluent enters the ion source, either electron or chemical ionization achieves a continuous production of ions. The ion beam produced from the content of the effluent is directed to the mass analyzer by one or more electronic lenses. The lenses also provide the ions with a relatively homogenous momentum or velocity, which is important for separation of the ions in the mass analyzer.

The extent of ionization can be affected by the ionization (or filament) current, since this parameter is related to the number of electrons emitted from the filament. The temperature of the ion source may also affect fragmentation due to the kinetic nature of the ionization processes.

In the case of some ion trap designs, the ions are produced in the mass analyzer itself.

4.3.1.1 Electron Ionization

In electron ionization (EI), a beam of electrons directly bombards the GC effluent. When an electron from the filament has a near-collision with the analyte, an electron is abstracted from the molecule, resulting in the formation of an energetic cation radical. The cation radical is called the "molecular ion." The most common electron beam energy is 70 eV, since an electron with this amount of energy causes ionization of essentially all organic molecules. The cation radical can undergo a predictable and relatively reproducible fragmentation, which results in a cation and a radical. The cations formed from bond cleavage reactions are called "fragment ions." The most abundant ion is called the "base peak."

4.3.1.2 Chemical Ionization

In the chemical ionization (CI) mode, a reagent gas is introduced into a specially designed ion source. The reagent gas is bombarded and ionized by the electron beam. The reagent gas ions react with the GC effluent and chemically ionize the sample. The most common reagent gases are methane, ammonia, and isobutane. The most common types of CI reactions resulting in positive ions are proton transfers. The appearance of a protonated molecular ion and any fragmentation is a function of the gas phase proton affinity of the analyte and the reagent gas. Chemical ionization is a low-energy process and there is

usually little fragmentation in CI as compared to EI. This can decrease the amount of identification information present in the spectrum.

Negative ions can be produced either by electron capture of thermalized electrons or reaction with proton-abstrating reagents such as O^- . The former has become a method of choice for molecules containing electronegative atoms, such as the halogens contained in the benzodiazepine drug class. Negative ion chemical ionization (NICI) should be clearly indicated, since by convention CI is used for positive ions.

The ion trap presents a unique situation for CI. Because ions are stored in the ion trap, the reagent gas ions have a much longer time to react with the analyte. Thus, a much lower pressure (concentration) of reagent gas is necessary to produce an ion. This has made possible the use of more chemicals, such as acetonitrile and tetrahydrofuran, as reagent gases. Unfortunately, if ionization takes place in the ion trap, there is no opportunity to generate thermalized electrons, and thus electron-capture NICI is not possible.

4.3.2 Mass Analyzer

The mass analyzer separates the ion beam generated in the source into its component parts on the basis of their mass-to-charge ratio (m/z).

4.3.2.1 Magnetic and Electrostatic Mass Analyzers

In a magnetic analyzer, ions produced continuously in the source are accelerated toward a magnetic field by a kilovolt potential such that all of the ions have the same kinetic energy. The magnetic field, which describes a sector of a circle, separates the ion beam into its components according to their momentum. Ion beams of specific m/z are separated from each other spatially, with each beam having a unique radius of trajectory which depends on the accelerating voltage and the magnetic field radius and strength. The ion beam with a selected m/z passes through an adjustable slit and impinges on an electron multiplier where it is detected. Mass resolution can be adjusted by changing the width of the slit. Ions of different m/z can be focused on the detector by changing the field strength of the electromagnet.

An electrostatic analyzer can focus charged particles according to their kinetic energy-to-charge ratio. Since kinetic energy is related to the mass of the particle, the trajectory of an ion of particular m/z is a function of the accelerating voltage, and the radius and strength of the electrostatic field. Thus ions of specific m/z can be focused on the detector by varying the electrostatic field strength. The combination of a magnetic and an electrostatic analyzer results in a "double-focusing" instrument, which has improved mass resolution since the two analyzers use complementary ion separation principles. Detection of an ion of particular m/z can be achieved by varying either the electric or the magnetic field strength. Ion transmission and focusing can be improved by additional sectors, which can result in "triple-focusing" instruments. All commercial sector mass analyzers are double- or triple-focusing instruments.

Sector mass analyzers are important for several reasons. First, they can achieve mass resolution sufficient to determine the atomic composition of an ion. Second, because of the high accelerating voltages, almost all of the ions formed in the source are transmitted to the detector. Thus, tuning in a sector instrument involves adjusting the magnetic and electrostatic fields to transmit the correct mass, but relative ion intensities are not adjustable. Mass spectra obtained from a sector instrument are thus considered to be the "gold standard." Finally, because of the efficiency of ion transmission, a sector instrument has the highest sensitivity and lowest limits of detection of the commonly used mass analyzers.

4.3.2.2 Quadrupole Mass Spectrometer

The most popular mass analyzer is the quadrupole mass spectrometer (QMS). Ions produced continuously in the ion source are accelerated into the aperture between two pairs of parallel rods. Direct-current (DC)

and radio frequency (RF) voltages on opposite pairs of rods deflect the ion in a plane perpendicular to its movement down the long axis of the rods. The DC voltage, RF voltage and frequency, and the geometry of the rods determine the m/z ratio of the ion whose stable trajectory allows passage through the filter. Ions of other m/z either collide with the rods or are pumped away by the vacuum system. A selected m/z ion can be transmitted through the rods by selecting the appropriate DC and RF voltages. By simultaneously increasing the DC and RF voltages in a fixed ratio, ions of increasing m/z are sequentially transmitted to the detector.

The voltages applied to accelerate the ions from the source must be low (e.g., a few volts) so that the ions spend sufficient time in the quadrupole field to obtain good mass resolution. This can result in decreased transmission efficiency for high mass ions. In addition, the absolute DC and RF voltages applied and their ratio during the scan influence the transmission efficiency and apparent width of the mass peak detected. Thus, in tuning a QMS in the scan mode, the trueness of the mass axis, the relative intensities of the ions, and the width of the mass peak (and thus mass resolution) can be adjusted. This has significant implications for mass spectral library searching and matching.

Transmission of a specific ion is a different process. Because the transmission of ions with other m/z is not of concern, DC and RF voltages are chosen which generate the largest chromatographic peak profile, usually by selecting a specific mass (e.g., 432.3). In addition, the DC/RF ratio can be selected to transmit more ions at the cost of decreased mass resolution. This will increase signal and may increase signal-to-noise ratio if no ions of similar m/z are in the background or matrix. It may be appropriate in this operating mode to select targeted tuning conditions that will not result in a spectral scan with accepted relative ion intensities for a reference compound like perfluorotributylamine (PFTBA).

4.3.2.3 Ion Trap

The ion trap also separates ions through their interaction with a quadrupole field generated by RF and DC voltages. The ion trap can operate in several modes: selected mass detection, selected mass storage, and selected mass ejection. In the selected mass ejection mode used on most commercially available ion traps, all ions formed during ionization are stored within a space surrounded by the ring and end-cap electrodes by application of an RF voltage to the ring electrode. A linear increase in RF voltage causes instability in the trajectory of ions of increasing m/z which results in their ejection from the ion trap. The ions are detected with an electron multiplier located outside of the ion trap. A variety of other electronic wave functions can be applied to the end-cap electrodes of the ion trap in order to improve performance or facilitate ion reactions such as mass spectrometry/mass spectrometry.

4.3.2.4 Time-of-Flight Mass Spectrometers

Time-of-flight (TOF) mass spectrometers separate ions based on the time required for the ions to travel a defined distance after acceleration in an electrical field. Higher-mass ions have lower velocities than lower-mass ions. One advantage of TOF MS is the extremely rapid scan speed that can be used in conjunction with "fast GC." The TOF analyzer is also capable of higher-mass resolution than the QMS, depending on the speed and handling of data acquisition. Due to the nature of the data acquisition, TOF MS instruments always operate in the full-scan mode.

4.3.3 Mass Spectrometry/Mass Spectrometry

Mass spectrometry/mass spectrometry (MS/MS) has been a relatively recent addition to analytical methodology. In this approach, a molecule is ionized and the molecular ion or a fragment ion is separated from other ions in a mass analyzer as described above. This precursor ion (also known as a "parent ion") is then focused into a collision cell where it undergoes an energetic collision with a target (or collision) gas. The collision energy is partially transformed into potential energy, which results in fragmentation to form product ions (also known as "daughter ions"). This process is called "collision-induced dissociation"

(CID). The product ions are then analyzed and either a spectrum of product ion mass abundances or a selected reaction product can be monitored. The main experimental parameters that affect the CID process are the collision energy, determined by voltages within the instrument, and the collision gas thickness (or pressure).

There are two fundamentally different approaches to MS/MS employing either kilovolt collision energies (magnetic/electrostatic mass analyzers) or volt collision energies (quadrupole and ion trap mass analyzers). Because of the energy differences, the spectra obtained are frequently different. High-energy CID applications often use H_2 as the collision gas. The collision cell is usually an open design, since the velocity of the precursor ion and the low mass of the collision gas result in relatively little scattering of the product ions. In contrast, most low-energy CID applications use argon (He in ion traps) as the collision gas, and must use a collision cell that refocuses the product ion beam. In the QMS, the collision cell is usually a set of RF-only quadrupole rods into which the collision gas flows. This approach is sometimes called "MS/MS in space." In the ion trap, the CID process occurs between the stored precursor ion and the helium bath gas. The collision energy is provided by application of a voltage waveform to the end cap electrodes. Product ions are stored with the same RF field, and ejected using a mass instability scan. Since the product ions are not separated spatially from the precursor ions, this technique is sometimes referred to as "MS/MS in time." In the ion trap, the collision gas thickness cannot be varied, but the time for reaction between the precursor and the collision gas can be controlled to vary the CID product ions.

Although MS/MS spectra can be obtained, there are no spectral libraries and the rational explanation of the fragmentation processes is not as well developed as for electron ionization MS. Thus, the main analytical application of GC/MS/MS to toxicology is selected reaction monitoring (SRM), where several product ions are selectively monitored as a function of time. Comparison of several product ion abundances between a reference standard and an unknown under the same GC/MS/MS conditions should allow the development of identification criteria.

It should be noted that introduction of another collision process and mass analysis could give rise to an MS/MS/MS spectrum or third-generation product ion for quantitation. It is also noteworthy that sector instruments are capable of monitoring metastable ions. In this approach, unstable ions produced in the ion source can fragment in the field free region between the ion source and the magnetic or electrostatic mass analyzer. Since the resulting metastable ion has the momentum of its precursor but the mass of the fragment, it gives a unique "apparent" mass. Monitoring of metastable ions can provide selectivity similar to that obtained from MS/MS. Because quadrupole and ion trap analyzers do not use the same mass separation principle, metastable ions are not observed.

4.3.4 Vacuum System

The dependence of the mass separation on the specific trajectory of an ion through the mass analyzer means that the ion cannot collide with any other molecules along its path. The observed pressure in a container is the result of molecules colliding with the walls of the container or each other. Therefore mass separation devices must be operated at a sufficiently low pressure that the path the ion takes from the source to the detector is shorter than the distance between collisions with other molecules. The relationship between pressure and the mean-free path is well established. The longer the path through the analyzer, the lower the operating vacuum requirements.

There are two exceptions to the requirement for high vacuum in a mass analyzer. It has been clearly demonstrated that the presence of a low-molecular-weight gas, such as helium, in the ion trap resulted in improved mass resolution. This is the result of the helium bath gas decreasing the energy dispersion of the ions in the trap, and moving them toward the center of the ion trap where they were more efficiently ejected. A similar finding of improved transmission efficiency and mass resolution was recently demonstrated for higher pressures in a collision cell for low-energy MS/MS.

The presence of large amounts of air from a leak can influence ionization efficiency, mass resolution, and system sensitivity. It can also cause electrical arcing and system failure, particularly in systems in which high voltages are used. In smaller amounts, it can increase the need for routine maintenance due to detrimental reactions with the filament, column, or other components of the system.

4.4 Data Acquisition

The use of a mass analyzer provides the ability to acquire and analyze data in a variety of ways (see Figure 1). In the scan mode, the components of the total ion beam having a specific m/z are focused sequentially on the detector. The width of the mass peak focused on the detector depends on the mass analyzer used and the computer control of the electronic signal processing. For quadrupole analyzers, from six to ten collections are made across a one-mass unit window. For double-focusing instruments, the ion optics and the width of the collection slit determine the mass resolution. Unless scan times must be minimized, the spectrum should be collected from above the ions observed from air (m/z 35 to 50), to well above the expected molecular weight of the sample. In the case of CI, the scan should begin above the m/z of the highest mass reagent gas ions. The resulting plot of relative ion abundance as a function of the mass-to-charge ratio is the mass spectrum. The ion with the highest abundance in a mass spectrum is termed the "base peak" and is normalized to 100%. It is common practice to report other ion fragment abundances as percentages of the base peak height. The molecular ion (M^+), that results from detection of the radical cation formed during EI, corresponds to the molecular weight of the compound. Observation of the molecular ion is an important contributor in the identification of a compound. In cases where the molecular ion is not observed, CI can be used to determine the molecular mass.

The application of the scan mode to drug analysis is frequently limited by the fact that the target compounds are present at a concentration too low to provide a reliable mass spectrum. For magnetic and electrostatic analyzers and the quadrupole mass spectrometer (QMS), selecting a specific m/z of interest and monitoring it for defined periods of time can enhance the signal-to-noise ratio. This approach is called "selected ion monitoring" (SIM) for single-mass analyzers and "selected reaction monitoring" (SRM) for tandem-mass analyzers.

The following summarizes current thinking regarding the use of the spectral scan versus SIM mode of operation.²

- Qualitative analysis: Whether it is better to scan the spectral mass spectrometer repetitively over the full mass range, or to monitor only selected ions, continues to be debated. Both modes of operation have their strengths and limitations, so it is unwise to adhere rigidly to only one mode of operation. A good quality full-scan mass spectrum generally provides the best qualitative identification; however, the SIM mode generally is more sensitive and less affected by potential interferences from coeluting compounds. The specificity (certainty of identification) of a SIM assay depends on many factors including: the number of ions monitored; the uniqueness of the monitored ions; the selectivity of the extraction procedure; the type of derivative; the efficiency of the chromatographic separation; and the selectivity of the method of ionization. A well-designed SIM assay can provide a very reliable method of identification. However, it may be difficult to evaluate the reliability of a SIM assay without personal experience with the method or access to data from the analysis of a substantial number of specimens. With either mode of mass analysis, the analyte's retention time (or better, its retention time relative to a reference standard) should agree well with the analyte's expected (relative) retention time.
- Quantitative analysis: The accuracy of quantitative measurements performed by GC/MS is highly dependent on the intensity of the analyte's ion current relative to the background ion current ('noise') intensity. Acceptable quantitative measurements can be obtained from reconstructed ion chromatograms, or total ion current chromatograms obtained under full-scan data acquisition, if a

relatively high concentration of analyte is present in the specimen. However, when analyte concentrations are in the low-nanogram/milliliter range, it is generally necessary to use selected ion monitoring to obtain sufficient ion current intensity for accurate quantitation.

For the purposes and definitions of this guideline, the quantitative analysis comments relate to the analysis of threshold compounds and some nonthreshold compounds, while the qualitative analysis comments relate exclusively to the analysis of nonthreshold compounds.

The ability to confirm the identity of compounds based on the relative intensity of SIM ions was originally investigated by Sphon.³ Using the spectrum of diethylstilbesterol as a model, a unique identification in a library of 30,000 compounds was obtained by comparing three ions from the spectrum at unit mass resolution with $\pm 30\%$ agreement between relative intensities of the base peak and $\pm 35\%$ agreement between relative intensities of the other two ions. Sphon concluded that three ions are regarded as a minimum, depending on the specificity of the ions monitored. It is important to note that the compound was not derivatized and that chromatographic resolving power of capillary GC was not available. More recent evaluation of these criteria against a larger database has confirmed the validity of the process.⁴

The fundamental basis of identification of a compound is the information content of the analytical data. Using this approach, other investigators have attempted to determine "selectivity indices" based on the inherent specificity of each step in the analytical procedure.⁵ The information content of GC/MS/MS has been investigated by Fetterolf and Yost.⁶

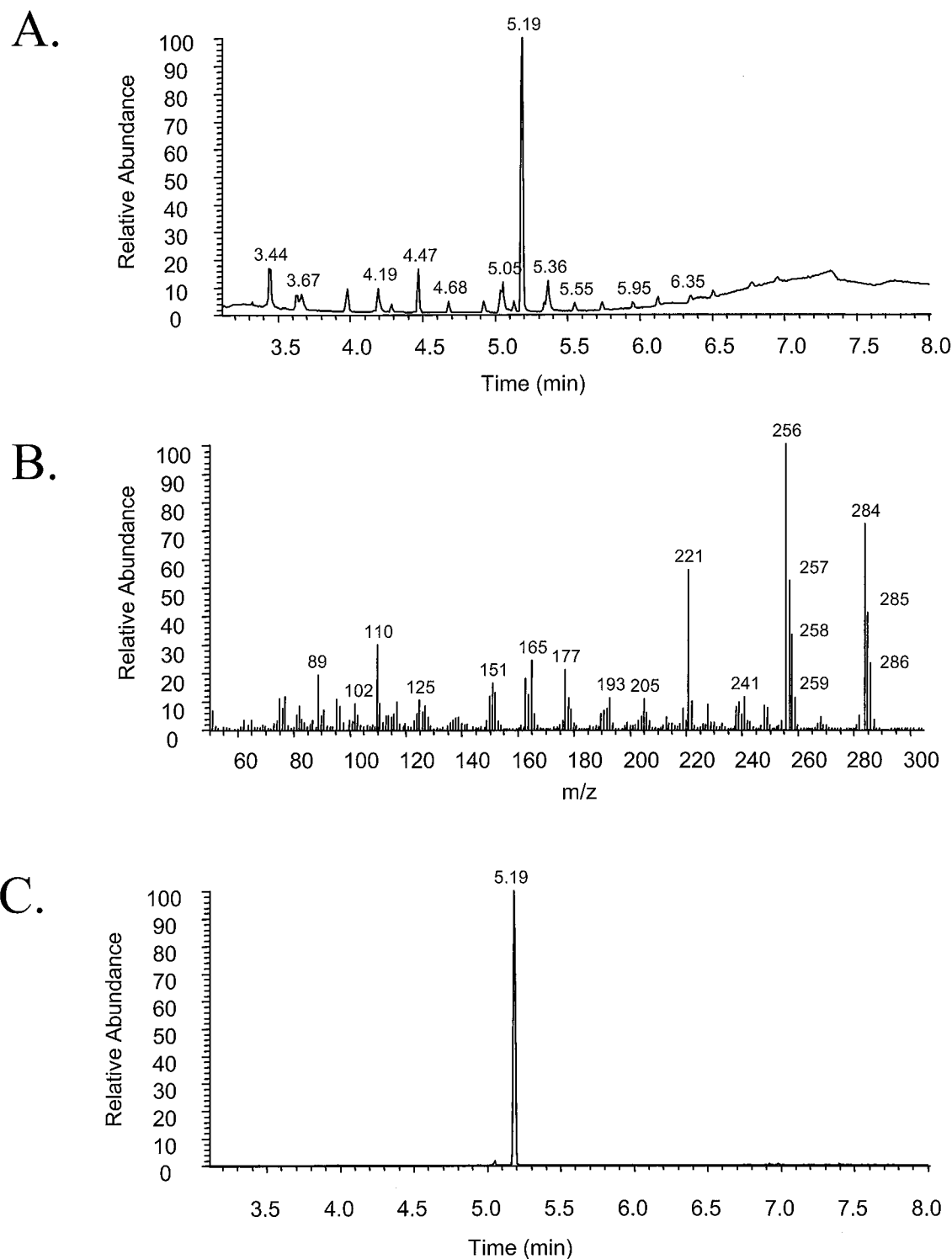


Figure 1. A) Representative GC/MS trace shown as a total ion chromatogram. B) Full scan mass spectra of diazepam, the peak that eluted at 5.19 minutes. C) Selected ion chromatogram (284 m/z) of the same data shown above.

5 Definitions^a

Accuracy//Measurement accuracy//Accuracy of measurement - 1) Closeness of the agreement between the result of a measurement and the accepted reference value of the measurand [analyte] **NOTE:** In the context of this guideline, accuracy comprises the following three concepts, described by the following three distinct ISO 3534-1 terms: **Accuracy** - Closeness of the agreement between a test result and the accepted reference value; **Trueness** - Closeness of agreement between the average value obtained from a large series of test results and an accepted reference value; and **Bias** - The difference between the expectation of the test results and an accepted reference value; **NOTE:** In general, the deviation/difference is based on replicate measurement using an accepted (definitive, reference, or designated comparison) method and the method being tested, and expressed in the units of the measurement or as a percentage.

Bias - See Accuracy.

Best measurement capability - The smallest uncertainty of measurement a laboratory can achieve for a stated calibration under specified laboratory conditions.⁷⁻¹⁰

Chemical ionization, CI - The formation of new ionized species when gaseous molecules interact with ions; **NOTE:** The process may involve transfer of an electron, a proton, or other charged species between the reactants. When positive ion results from chemical ionization (CI), the term may be used without qualification; when a negative ion results, the term “negative ion chemical ionization” can be substituted. Specifics relating to ionization should be given, e.g., if negative ions are formed from sample molecules via resonance capture of thermal electrons generated in a CI source, this should be specified.¹¹

Collision-induced dissociation, CID - An ion/neutral process wherein the (fast) projectile ion is dissociated as a result of interaction with a target neutral species; **NOTE:** This is brought about by conversion during the collision of part of the translational energy of the ion to internal energy in the ion.¹¹

Drug - Any substance which when absorbed into a living organism may modify one or more of its functions.¹²

Drug of abuse - Drug used for a nontherapeutic purpose.¹²

Electron ionization, EI - Ionization of any species by electrons; **NOTE:** Electrons and photons do not “impact” molecules or atoms. They interact with them in ways that result in various electronic excitations including ionization. For that reason it is recommended that the terms “electron impact” and “photon impact” be avoided.¹¹

Electrostatic analyzer - A velocity-focusing device for producing an electrostatic field perpendicular to the direction of ion travel (usually used in combination with a magnetic analyzer for mass analysis); **NOTE:** The effect is to bring to a common focus all ions of a given kinetic energy.¹¹

Extracted ion chromatogram, EIC - Describes the processing of data from a mass spectrometer in which the ion current at one (or several) m/z values acquired in the spectral scan mode are selected and displayed as a function of time.¹¹

Ion current - The intensity of an ion beam produced in the source, passed through the mass analyzer, and measured by the detector.¹¹

^a Some of these definitions are found in NCCLS document NRSL8—*Terminology and Definitions for Use in NCCLS Documents*. For complete definitions and detailed source information, please refer to the most current edition of that document.

Ion ratio - The ratio of signal intensities at two m/z values, usually expressed as a percentage; **NOTE:** The ratio may be determined from the ratio of chromatographic peak areas or peak heights, or may be calculated from a single mass spectrum.¹¹

Limit of detection, LOD - 1) The smallest amount or concentration of analyte that can be distinguished from background at a stated confidence level; **NOTE:** For GC/MS or GC/MS/MS confirmation analysis, the compound must also satisfy identification criteria in order to be deemed detected; **2)** the lowest amount of analyte in a sample which can be detected but not quantified as an exact value.⁷⁻¹⁰

Limit of quantitation, LOQ - The lowest amount of analyte in a sample that can be quantitatively determined with {stated} acceptable precision and {stated, acceptable} bias under stated experimental conditions.⁷⁻¹⁰

Magnetic analyzer - A direction-focusing device that produces a magnetic field perpendicular to the direction of ion travel; **NOTE:** The effect is to bring to a common focus all ions of a given momentum with the same mass-to-charge ratio.¹¹

Mass spectrum - A spectrum obtained when ions (usually in a beam) are separated according to the mass-to-charge ratios of the ionic species present; **NOTE:** This plot is a graphical representation of m/z versus the measured abundance information.¹¹

m/z - An abbreviation used to denote the dimensionless quantity formed by dividing the mass of an ion by the number of charges carried by the ion; **NOTE:** It has long been called the “mass-to-charge ratio” although m is not the ionic mass nor is z a multiple of the electronic charge, e^- .¹¹

Nonthreshold substance - A compound for which detection and identification of any amount of compound is considered to be a “positive” or “present.”

Peak resolution, R_s - The separation of two peaks ($t_{R2} > t_{R1}$) in terms of their average peak width at base (w_b): $R_s = 2(t_{R2} - t_{R1}) / (w_{b1} + w_{b2})$; **NOTE:** In the case of two adjacent peaks it may be assumed that $w_{b1} = w_{b2}$, and thus, the width of the second peak may be substituted for the average value: $R_s = (t_{R1} - t_{R2}) / w_{b2}$.¹³

Plate number - A number indicative of column performance, calculated from the following equations which depend on the selection of the peak width expression:

$$N = 5.545 (V_R / w_b)^2 = 5.545 (t_R / w_b)^2$$

In these expressions the units for the quantities inside the brackets must be consistent so that their ratio is dimensionless: i.e., if the numerator is a volume, then peak width must also be expressed in terms of volume.¹³

Precision - In the context of this guideline, precision is defined the way ISO 3534-1 defines **Uncertainty** (defined below).

Precursor ion - An electrically charged molecular moiety which may dissociate to form fragments, of which one or more may be electrically charged, and one or more are neutral species; **NOTE:** A precursor ion may be a molecular ion or an electrically charged fragment of a molecular ion.¹¹

Product ion - An electrically charged product of a reaction of a particular precursor or parent ion; **NOTE:** In general, such ions have a direct relationship to a precursor ion and indeed may relate to a unique state of the precursor ion.¹¹

Radical ion - An ion containing an unpaired electron that is thus both an ion and a free radical; **NOTE:** The presence of the odd electron is denoted by placing a dot alongside the symbol for the charge.¹¹

Selected ion monitoring, SIM - Describes the operation of a mass spectrometer in which the ion currents at one (or several) selected m/z values are recorded rather than the entire mass spectrum; **NOTE:** The use of terms "multiple ion detection (MID)," "multiple ion (peak) monitoring (MPM)," and "mass fragmentography" are not recommended.¹¹

Selected reaction monitoring, SRM - Describes the operation of a tandem mass spectrometer in which the product ion currents at one (or several) selected m/z values are recorded rather than the entire mass spectrum.¹¹

Sensitivity (analytical) - Change in the response of a measuring system or instrument divided by the corresponding change in the stimulus (e.g., analyte concentration).^{7,8,9,10}

Analytical specificity - In Quantitative Testing, the ability of an analytical method to determine only the component it purports to measure or the extent to which the assay responds only to all subsets of a specified analyte and not to other substances present in the sample.

Tandem mass spectrometry, MS/MS - A technique in which an ion at a particular m/z value is isolated in one mass analysis procedure, caused to undergo fragmentation, and the products of the fragmentation process analyzed in a second mass analysis procedure; **NOTE:** The use of the abbreviation "TMS" is discouraged.¹¹

Threshold substance - A compound for which a concentration has been specified, on either an administrative or scientific basis, above which the compound is deemed to be "positive" or "present" and below which the compound is deemed to be "negative" or "not detected."

Total ion chromatogram, TIC - The sum of all the separate ion currents carried by the different ions contributing to the mass spectrum plotted as a function of time.¹¹

Trueness - See Accuracy.

Uncertainty - An estimate attached to a test result which characterizes the range of values within which the true value is asserted to lie.

6 Method Validation

All methods must be validated in a manner appropriate for the final use of the data generated by the method. A GC/MS confirmation method need not be specific for a single compound. When multiple drugs and metabolites are confirmed in the same method, validation data must be available for each compound. For threshold compounds and nonthreshold compounds for which quantitative data is determined, validation of the procedure should include all of the following parameters. Nonthreshold substances for which only detection and/or identification are reported should have specificity, limit of detection, and robustness determined, when possible.

Validation studies should be repeated if any change is made to the procedure that could affect the results.

6.1 Specificity

An investigation of specificity should be conducted during the validation of the assay. The confirmation test should be able to discriminate between compounds of closely related structures that are likely to be present. The specificity of an assay can be established by obtaining negative results in a suitable number

of known negative samples, combined with positive results in either known positive samples or negative samples spiked with a traceable reference material. The specificity of the assay may also be determined by comparison of results to those obtained by a well-characterized reference method.

In addition, the assay should be performed with compounds structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of potentially interfering materials should be based on reasonable scientific judgment with a consideration of the interferences that could occur. Elimination of a compound from consideration as an interference may also be based on scientific judgment and the structure of the potential interference.

It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte. For identification of nonthreshold substances, where the screening procedure might have similar characteristics, this may be an important consideration. In this case, a combination of two or more analytical procedures (different extraction, different chromatographic conditions, different derivative, etc.) is recommended to achieve the necessary level of specificity.

6.1.1 Specific Recommendations for Nonthreshold Compounds

In circumstances where a traceable reference standard is not available (e.g., a metabolite of a drug), the use of either a biological fluid obtained after a documented ingestion of the drug of interest or a metabolite isolated from a biological sample is acceptable as a secondary reference material. The compound should be characterized to verify its identity. Comparison of GC/MS data obtained from analysis of the biological fluid or isolate in the laboratory to mass spectra published in a reputable scientific journal is acceptable verification.

The secondary reference material should be analyzed in the same analytical run as the unknown.

6.2 Trueness

Trueness is the ability of the analytical procedure to measure the true concentration or amount of analyte. The measure of trueness is called bias. There are several methods of determining bias: repeated measurement of an analyte of known purity (e.g., a reference compound); or comparison of the measured results of the proposed analytical procedure to those of a second, well-characterized procedure whose trueness is stated or defined. Comparison of measured results of the proposed analytical procedure to those obtained as a mean of a proficiency testing survey can be used to measure bias but should be used with caution because proficiency testing results are subject to non-specific and unpredictable matrix interference. Data for determining the bias of a procedure may be obtained concurrently with precision, linearity, and specificity data.

Bias should be assessed over the entire range of expected concentrations. A mean of at least three determinations at each concentration should be used to assess bias. The bias of the method should be determined for at least three concentrations. It is recommended that one of the concentrations be near the threshold concentration, where appropriate.

These measures of trueness should be evaluated at least every twelve months or when major maintenance is performed.

6.2.1 Carryover

Contamination of a sample with residual drug from another sample or standard seriously degrades the accuracy of the analysis. Procedures should be developed and instrumental conditions should be selected to minimize or eliminate carryover. Carryover can originate from the injection syringe, wash reservoir solvents, injection port, or column. The extent of carryover should be determined under specified

conditions. In the event of potential carryover during an analytical run, the procedure should contain specific instructions for eliminating carryover and obtaining an accurate result. An example of such a procedure would be inclusion of a blank injection prior to injection of each sample of interest.

6.3 Precision (Repeatability and Reproducibility)

Repeatability is the ability of the method to provide closely similar results for the same measurand under the same conditions of measurement in a short time frame. Repeatability is sometimes called within-run imprecision. Reproducibility is a measure of imprecision when the conditions of measurement vary across time, technicians, or laboratory equipment. There are within-lab and between-lab measures of reproducibility. Within-lab reproducibility can also be called between-run reproducibility. Reproducibility between laboratories is frequently assessed by means of an interlaboratory trial or by measurements of a control material over time.

6.3.1 Specific Recommendations for Threshold Compounds

Repeatability (within-run precision) should be assessed at a minimum of three concentrations covering the specified range for the procedure using a minimum of three replicates at each concentration.

Reproducibility should be estimated when multiple instruments and analysts are used in a procedure.

Both within-run and between-run precision can be determined simultaneously using appropriately designed experiments with multiple determinations on each of several days. Appropriate analysis of variance experiments can identify the relative contribution of several sources of variability in a method.

These measures of precision should be evaluated at least every twelve months or when major maintenance is performed.

6.4 Linearity

Linearity should be established by visual evaluation of a plot of peak height or area (or in the case of an internal standard method, peak height ratio or area ratio) as a function of analyte concentration. For establishing linearity, a minimum of five concentrations is required. Linearity should be established across a range of concentrations that reflects those expected in the biological matrix.

If there is a linear relationship, test results should be evaluated by calculation of a regression line by the method of least squares. Data from the regression line may also be useful to provide mathematical estimates of the degree of linearity. The residuals from the regression line as a function of concentration should be evaluated for a random distribution to support a linear relationship. The correlation coefficient can have serious limitations, particularly if the concentration range investigated is greater than one order of magnitude.¹⁴ Despite this limitation, the correlation coefficient is a useful indicator of linearity if it is greater than 0.998.¹⁵ An alternative to this is to plot the response factor (peak area or height divided by concentration) as a function of concentration, which should be a constant. A more robust and technically difficult method has been described.¹⁶

The linear range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, bias, and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The linear range should be re-evaluated every twelve months or when major maintenance has been performed.

The standard concentrations for the calibration curve should be selected to minimize the uncertainty of the linear regression line near the threshold concentration. The best precision (lowest uncertainty) about

the regression line occurs at the mean of the standard concentrations. Where practical, the standard concentrations for the analysis should be selected so that their mean lies near the threshold concentration.

For more information on evaluating an instrument or quantitative analytical method on the basis of the manufacturer's linearity claim, see the most current version of NCCLS document EP6—*Evaluation of the Linearity of Quantitative Analytical Methods*.

6.5 Limit of Detection

A variety of methods have been proposed to determine the limit of detection (LOD) or best measurement capability.¹⁷⁻²⁴ Most of these methods rely on signal strength from a single channel of analytical data and the ability to distinguish a difference between the signal and the background at some level of confidence.¹⁷ The LOD depends strongly on the matrix background "noise" as well as instrumental noise. In determining the LOD, the method should consider the probability of false-positive and false-negative results as well as true positive and negative results.

GC/MS provides multichannel detection capability by virtue of the simultaneous detection of multiple m/z ratio ions as a function of retention time. As a result, GC/MS data contain both identification and quantification information. For the purposes of this guideline on confirmation, detection requires both a signal discernable from the background and satisfaction of identification criteria used for the method.

Historically, two methods have been used to determine the LOD in toxicology: the statistical approach and the empirical approach.²⁴ The statistical approach requires analysis of ten replicates of a known negative sample, measurement of the system noise, and calculation of a concentration corresponding to three times the matrix noise level. The empirical approach requires analysis of a series of decreasing concentrations of drug in the biological matrix. The LOD is the concentration at which it is no longer possible to detect and identify the drug.

Other methods have included the minimum detectable limit,²² and probability of detection.²³ For qualitative methods, information theory has been applied to estimate a probability of identification.^{25,26} In general, methods to determine the probability of identification require more extensive studies because nonparametric statistical approaches are used.

The LOD should be verified every twelve months or when major maintenance is performed.

6.5.1 Recommendations for Threshold Compounds

The empirical method is recommended for determining the LOD. Serial dilutions of a sample with a known concentration should be made using an appropriate biological matrix as diluent. Given the role of the matrix "noise" in detection, more than one biological matrix diluent may be desirable. The LOD is the lowest concentration where both detection and identification criteria are met. The LOD should be verified by triplicate analysis of a sample where all replicates meet criteria for detection and identification. Any bias between the concentration measured and that expected should not be considered.

6.5.2 Specific Recommendations for Nonthreshold Compounds

The empirical method may be applied to estimate the LOD for nonthreshold compounds that are not quantified. In the case where no reference standard is available, a sample for which the concentration has been estimated may be used. The procedure should clearly state the conditions under which the LOD was determined.

6.6 Limit of Quantitation

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be quantitatively detected with a stated acceptable uncertainty and bias under stated experimental conditions. For GC/MS confirmation analyses, the concentration measured in the appropriate matrix to which a suitable reference material has been added should be within $\pm 20\%$ of the expected value (bias) with a coefficient of variation not greater than 20%. The imprecision of the analysis should be determined using a minimum of three measurements.

The LOQ should be verified every twelve months or when major maintenance is performed.

6.7 Robustness

Robustness is the reliability of an analysis with respect to variations in method parameters. If the results are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. A set of system suitability parameters (e.g., resolution test, minimum signal or signal-to-noise ratio) should be developed to ensure that the validity of the analytical procedure is maintained whenever used (see Section 7).

Typical GC/MS procedure parameters to be evaluated in determining robustness may include:

- stability of analytical solutions;
- hydrolysis conditions (e.g., solutions, temperatures);
- different lots of extraction materials;
- derivatization conditions (e.g., solutions, temperatures);
- stability of analytes after derivatization;
- injection parameters (e.g., volume, temperature, flows);
- different instruments;
- different columns (different lots and/or suppliers);
- temperature (isothermal or programmed) variation;
- flow rate variation; and
- different analysts.

7 Routine Instrument and Method Performance Verification

A GC/MS confirmation method should contain, or refer to, a procedure for documenting proper performance of the analytical instrumentation prior to analyzing any samples. In addition to verifying instrument function daily, quality control samples should be included with each batch of samples to ensure method performance. The selection of the quality control sample should reflect the intended purpose of the analysis.

7.1 Gas Chromatograph

Capillary column gas chromatography is used for the majority of separations used for GC/MS confirmation work. The primary criteria for suitable chromatographic performance are retention time reproducibility, peak narrowness and asymmetry, peak resolution, and signal-to-noise ratio (or signal) at a specified concentration. It is important to note that the peak criteria reflect injector, column, and detector performance at the time of the confirmation. The compounds selected for use in evaluation of the system should be suitable for that purpose. The chromatographic system performance verification mixture should be run at least once each day and must be run after a system repair or modification prior to analysis of any specimen.

Prior to analyzing samples, the chromatographic system should be evaluated for adequate performance. Peak narrowness and asymmetry are good measures of the efficiency of the chromatographic system. The performance verification mixture should include an appropriate compound for which these parameters are monitored. Peak narrowness should be measured by the plate number measured at the half-height of the peak profile. Minimum acceptable values for these criteria must be included in the quality assurance portion of the procedure.

Peak asymmetry is generally measured as the ratio of the distance from a perpendicular dropped from the peak apex to the peak profile at a point 10% of the distance from the baseline to the peak apex. A perfectly symmetrical peak has an asymmetry value of 1.0. A peak for which the distance to the trailing edge is longer than that to the leading edge has a value greater than one and is called "tailed." A peak for which the distance to the leading edge is longer than that to the trailing edge has a value less than one and is called "fronted." The procedure must define an acceptable range of values for peak asymmetry.

A pair of appropriate compounds should be included in the performance verification mixture that assesses the ability of the system to clearly separate the compounds. The test compounds should represent the type of compounds that are being separated in the analysis. The procedure should define an acceptable range of values for resolution. The resolution may be expressed either as the defined calculation for resolution (R_s) or as the depth of valley between the peak apices. For the purposes of this discussion, the valley point height should be measured as a function of the height of the smaller peak. The minimum resolution between an adjacent pair of peaks should be at least 1.25 or approximately 10% valley/peak ratio. The rationale for recommending this chromatographic resolution is that it has been shown that peak height measurements maintain a deviation of less than 1% from the true height (accuracy) at this resolution over a 100-fold ratio of peak sizes. A similar accuracy in peak area can be obtained over a 32-fold range.²⁷

System response to a known concentration should also be documented. One or more compounds in the system performance mixture should be evaluated for either absolute signal or signal-to-noise ratio at a specified concentration. The internal standard may also be used for this assessment of system function.

7.2 Mass Spectrometer

7.2.1 Mass Axis and Abundance Calibration

A reference compound, such as perfluorotributylamine (PFTBA), decafluorotriphenylphosphine (DFTPP), or other appropriate compound, must be used to calibrate the mass axis of the mass analyzer. In the case of QMF and ion trap instruments, the reference compound is also used to set the relative abundance of selected ions as part of the tuning process.

7.2.1.1 Acceptance Criteria for the Tuning Compound Perfluorotributylamine

The laboratory must establish acceptable ranges for the relative abundance of ions m/z 69, m/z 219, and m/z 502. Widely accepted specifications for EI QMF instruments are: m/z 69, 70 to 100% of the base peak; m/z 219, greater than 20% of the base peak; and m/z 502, greater than 2% of the base peak. These specifications were developed primarily to minimize the effect of tuning on computerized library spectral matching algorithms. Manufacturers' recommendations should be considered in establishing the relative abundance ranges. Acceptance criteria should also include inspection of the mass peaks for symmetry and the absence of precursors. There should also be resolution between each major ion and its ^{13}C -isotope mass peak. In addition, it is important to monitor the consistency of the ion abundance and instrument settings between days. If an unacceptable change is observed, the underlying reason for such a change should be determined, and the problem corrected. All corrective actions must be documented.

Target tuning for high-mass compounds or selected ion monitoring (SIM) operation is acceptable. Although this approach to tuning precludes interlaboratory comparisons of resulting spectral data, the

increase in sensitivity is usually advantageous. Methodological considerations may require that the m/z 502 ion relative abundance be as high as 50%.

When tuning tandem mass spectrometry instrumentation, the manufacturer's recommendations should be considered.

7.2.1.2 Acceptance Criteria for Alternative Tuning Compounds

Alternative tuning compounds may be used for specific purposes, such as performance enhancements for measurements associated with high molecular weight compounds, for negative ion detection, or for advanced modes of operation such as tandem mass spectrometry (MS/MS). The laboratory must establish acceptable ranges for the relative abundance of ions based on criteria in the peer-reviewed literature or by the instrument manufacturer.

7.2.2 Air Leak Criteria

Proper operation of the mass spectrometer also requires that minimal amounts of air (and water) are contained in the system. The presence of large amounts of air from a leak can influence ionization efficiency, mass resolution, and system sensitivity. The laboratory must establish acceptance criteria for the presence of these compounds.

The recommended approach is to monitor the ions produced from the constituents of air itself. In a leak-free system which has been under vacuum for some time, nearly all the components of air will be pumped away, but water will be observed at m/z 17 and 18 due to the difference in the pumping efficiency for water. If the abundance ratio of m/z 28 (N_2^+) to m/z 18 (H_2O^+) is less than 0.5, the system is free of leaks. If the ratio of m/z 28 to m/z 18 is greater than 2 to 1, there is an air leak. In the case of a leak, the ratio of m/z 28 to m/z 32 (O_2^+) will also be about 3, reflecting the composition of air. Under these circumstances, the leak should be located, fixed, and the repair documented. If the ratio of m/z 28 to m/z 18 is between 0.5 and 2, the situation should be monitored. Note that a large leak may result in saturation of the m/z 28 peak and give falsely low ratios. A noticeable increase in system pressure as well as the presence of significant amounts of m/z 14 (N^+) and m/z 16 (O^+) relative to m/z 18 are indications of a severe air leak.

Some manufacturers have elected to use the abundance of the m/z 28 ion from N_2 relative to m/z 69 from PFTBA tuning reference compound as an indication of an air leak. This approach is based on a consistent flow of PFTBA from the calibration gas vial. It is frequently specified in the range of 10 to 20%. The laboratory may choose to follow the manufacturer's recommendation on the specific relative abundance of these ions as a daily monitor for an air leak.

7.3 Calibration

7.3.1 Procedures

7.3.1.1 Single-Point Calibration

If the purpose of the analysis is to demonstrate that the concentration of a substance is greater than a threshold value, a single calibrator analyzed contemporaneously with a concentration equal to the threshold value may be used. Since the purpose of the measurement is to convey a degree of confidence that the value is greater than the threshold, it is recommended that the margin by which the sample exceeds the threshold be documented to have statistical significance.

7.3.1.2 Multipoint Calibration Curve

When a linear calibration curve is observed, the use of appropriate standard concentrations can maximize the precision of the curve at the threshold concentration. The narrowest point for the confidence band about a linear regression line occurs at the mean value of the ordinate (x) values. Where practical, concentrations for standards should be chosen so that the threshold concentration is at or near the mean value of the concentrations analyzed for the calibration.

The concentrations of the standards should be computed from the linear regression line and should agree within $\pm 20\%$ of the nominal value. If more than three concentrations are used to establish the curve, one standard may be dropped for not meeting acceptance criteria. Standards may not be dropped to improve the curve fit, to bring control results into acceptance range, or simply because its concentration is outside the target range. There must be a logical reason for dropping a standard (e.g., poor recovery, interfering peak, incorrect integration) and it must be documented.

7.3.1.3 Historical Multipoint Calibration Curve

If the standard curve has been documented to be linear and stable over a specified time interval, a historical calibration curve may be used to determine the concentration. In this case, at least two controls or standards should be analyzed (one of which should be at the threshold concentration), and the concentrations and ion ratios must be within $\pm 20\%$ of the initial measurements.

7.3.2 Internal Standard

It is advisable to use an internal standard, added prior to any extraction steps, to assess the performance of sample preparation and instrument function. For mass spectrometric detection, the use of a compound labeled with a stable isotope such as deuterium is recommended. If a deuterated internal standard is not available, or if a number of compounds are confirmed in the same procedure, it is acceptable to use a structurally related compound. The internal standard must undergo any derivatization reactions used in the procedure.

7.4 Quality Control/Quality Assurance

An important component of assuring analytical quality is an active quality assurance program. The program should encompass the range of compounds to be expected in the analysis, although the number of compounds confirmed and the number of analytical batches analyzed may preclude frequent assessment of every compound. The quality control samples must constitute at least 10% of the samples run in an analytical batch. Blind quality control samples should be included in the program which challenge not only the analytical portion of the assay, but also clerical and urine integrity testing if this is included as a part of the confirmation analysis.

Specific criteria must be established for acceptance or rejection of the quality assurance specimens. For quantitative assays, a comprehensive set of criteria, such as Westgard's rules, should be used if the volume of sample batches is sufficient to establish the precision characteristics of the assay. An acceptable alternative is to use $\pm 20\%$ of an established mean. Procedures should state the actions to be taken if quality assurance specimens fail to meet established criteria. Regular, active evaluation of quality assurance results must be documented.

Negative control samples should be routinely run, and must be negative in order to accept analytical batch results. It is recommended that a negative control sample be run immediately before each presumptively positive sample to ensure that there is no carryover from standards or controls.

If the confirmation procedure requires hydrolysis to remove glucuronide, sulfate, glutathione, or other conjugates, the hydrolysis procedure should be routinely assessed for completeness. It is recommended that the hydrolysis control, where available, be included with each analytical batch.

7.4.1 Specific Recommendations for Threshold Compounds

Quality assurance samples must be used near the analytical threshold. The concentration should be such that a positive finding is always achieved. A concentration 20 to 25% above the threshold is frequently used to achieve this goal. The positive control should be used for each threshold if more than one threshold is used in a single assay. A negative control with a concentration 20 to 25% below the threshold may be used to document the ability to determine a negative result near the threshold value. In cases where quantitative results are provided, a control sample may be used to document the linear range of the assay.

7.4.2 Specific Recommendations for Nonthreshold Compounds

Positive and negative control materials should be used in each analytical batch to document the presence and absence of the compound to be identified. It is recommended that the positive control contain a concentration comparable to that of the sample.

8 Identification Principles

In confirmation analyses, the objective is to identify the compound within a determined level of confidence. It has been suggested that the level of confidence for legal proceedings be 1 in 10,000 to 1 in 100,000.²⁸ Analysis procedures or combinations of procedures with greater information content increase the level of confidence in an identification.

8.1 Gas Chromatograph

Due to its high-peak resolution capacity, the use of capillary gas chromatography in conjunction with mass spectrometry significantly improves the information content of the analysis. It should be noted that co-elution of a peak with a compound of known structure does not prove identity, but rather demonstrates consistency with the identity of the known compound. The retention time of the peak due to the presumed compound should elute within $\pm 1\%$ or ± 0.2 minutes (whichever is smaller) of the retention time of a peak of a contemporaneously analyzed standard.

Chromatographic peak overload of matrix components or analytes may cause a shift in retention time. If a deuterated internal standard is used, the analysis may be accepted if the retention time difference between the internal standard and the proposed analyte is the same as the difference observed for a control or standard not exhibiting the peak overload condition. If no deuterated internal standard is used, standard addition of the suspected compound may be used to document co-elution. In this approach, an amount of pure standard comparable to that in the original analysis is added to the sample, and the sample is analyzed using the normal procedure. To document identity, only the chromatographic peak presumptively identified should be increased, and the peak profile at half-height should be within $\pm 10\%$ of the original peak width.

The peak of interest should be separated from any other peaks so that there is resolution of at least 1.25 or a valley of at least 90% between peak maxima (for equal-sized peaks). In GC/MS, the additional information obtained from mass-selective detection can be used to enhance chromatographic resolution. Deconvolution or peak purity algorithms can be used to resolve partially co-eluting peaks.²⁹ This can also be done manually by inspecting the consistency of mass spectra across the peak profile.

8.2 Mass Spectral Identification

A mass spectrum of an unknown component may provide a definitive identification. For this reason, application of GC/MS under appropriate conditions is considered the "gold standard" for identification. As mentioned above, acquisition of a complete mass spectrum is preferable to acquisition of selected ions. In those cases where high-quality full or partial spectra cannot be obtained, selected ion monitoring is acceptable.

8.2.1 Full-Scan Acquisition

The mass spectrum should be acquired from m/z 40 to at least 100 mass units above the expected molecular mass ion. In applications where ions from the derivatizing reagent dominate the spectrum, it is acceptable to acquire data from just above the mass of the derivatizing reagent. The acquisition of partial spectra may also be acceptable. In this case, the partial spectrum should be compared to that of a contemporaneously analyzed standard. Use of a spectral matching algorithm to compare a partial spectrum to a full spectrum is not acceptable.

Either the absence or presence of ions at a particular mass is informative. If manual inspection and identification of compounds is performed, the laboratory must develop guidelines for comparability of an unknown spectrum to that of a contemporaneously analyzed standard. Some typical guidelines may include: presence in the unknown spectrum of all mass ions with a relative abundance greater than 15% in the reference spectrum; agreement of ion intensities within $\pm 20\%$ relative abundance; absence of ions beyond the molecular ion cluster giving rise to a significant ion in spectrum (e.g., M-90 for TMS); absence of any ions greater than 50% relative abundance in the unknown spectrum that do not occur in the standard spectrum; relative abundance of isotopes in molecular ion cluster consistent with contemporaneous reference material or theory; and the absence of illogical mass losses in the spectrum. Inspection of the consistency of spectra across the peak profile may be helpful. The exercise of scientific expertise and judgment is appropriate.

8.2.2 Computer-Based Spectral Library Matching

The library search mode used in most laboratories is the identification search or reverse search. The search algorithm is designed to assess the presence of a target compound from a database of spectra in a spectrum obtained from a chromatographic peak. It is assumed in these algorithms that ions not in the reference spectrum are from impurities, and these ions are ignored. Other factors may also be applied to the experimental spectra in order to improve the ability of the algorithm to match a spectrum in the library database. The two most common commercially available algorithms are the probability-based matching (PBM) approach³⁰ and the dot product approach.^{b 31}

The reliability of computer-aided mass spectral matching to a library spectrum is dependent on a number of factors including: the search algorithm; the quality of the experimental spectrum; the presence of the spectrum in the library; the quality of the library spectra; the use of complete versus condensed library spectra; and instrumental factors such as tuning and source temperature. Decreasing analyte concentration makes all ions less abundant and more variable, decreasing the quality of the spectrum and the confidence index for the match.³⁰

The laboratory must establish criteria for acceptance of compound identification based on the "spectral match" quality. A spectral match factor of greater than 95 is generally associated with a correct identification. Since the match factor does not guarantee identification, all spectral library matches should

^b Distributed by the National Institute of Standards and Technology (NIST) and others.

be reviewed by a qualified scientist. It is recommended that for spectral match quality of between 75 and 95, the spectra should be evaluated by a qualified scientist for potential compound identification.

8.2.3 Selected Ion Monitoring Acquisition

As mentioned above, it has been demonstrated that the relative ion abundance ratios of three or four ions can be used to identify a compound. It should be noted that the ions must be diagnostic of the structure of the compound. In general, ions of higher abundance are selected due to their better reproducibility and lower limit of detection. Structurally significant ions should be selected over ions that have greater abundance but are not diagnostic. If sufficiently abundant, one of the ions selected should be the molecular ion (M^+). In any case, not more than one ion should be from any derivatization moiety.

There has been some confusion in the literature about the agreement between the observed ion ratios due to the fact that both the relative abundance and the variation in observed ratios have been described in percentage. At high relative abundance, this is of little consequence. For low relative abundance ions, there is a significant difference in outcome. For example, if a $\pm 20\%$ variation is computed at a relative abundance of 80%, a range of observed relative abundances between 64% and 96% would be accepted. The same criteria applied to an ion of relative abundance 10% results in an acceptable range of only 8 to 12%. If a $\pm 10\%$ abundance criteria is adopted, the corresponding ranges are 70 to 90% and 0 to 20%.

The laboratory must define what its identification criteria are for ion ratio matching. For the unknown compound, two ion ratios (three ions monitored) must be within the acceptable range for identification. The ion of largest abundance is frequently used for quantitative purposes and is referred to as the "quantification or quant ion" while the other two ions monitored for identification purposes are referred to as "qualifier or qual ions." For the internal standard, one ion ratio must be within the acceptable range (two ions monitored). It is recommended that the unknown compound have ion ratios within $\pm 20\%$ variation of the ion ratios measured in a contemporaneously analyzed standard if the relative abundance is greater than 20%, but a $\pm 5\%$ abundance range should be accepted when the measured relative abundance is less than 20% (e.g., $60 \pm 12\%$ and $18 \pm 5\%$ relative abundance). In addition, if the calculated acceptance range includes zero or a negative relative abundance, the lower end of the acceptance range should be 1% (i.e., the ion must be present).

The ion intensities for identification may be obtained from integrated peak height or peak area ratio measurements or from the ratios within a single acquisition. The ratio may be computed directly from the heights or areas. In order to be consistent with identity, all ions from the same peak profile must appear within one mass spectral acquisition of each other at the apex. If more than one standard is analyzed contemporaneously, the ion ratio acceptance range may be computed from a single standard, an average of standards, or a weighted average of standards. The method of computation must be documented. It is generally not acceptable to use different standards or different methods of computation for different specimens in the batch.

If screening procedure is also GC or GC/MS, and if less than three structurally characteristic ions are available, it is recommended that a second chemical technique or method be used. A change in derivatization chemistry can be considered a different technique.

8.2.4 Chemical Ionization

Chemical ionization mass spectra are characterized by less fragmentation but greater sensitivity than electron ionization. Since the ionization process is based on the kinetics of chemical reactions, the reproducibility of ion-relative abundances is somewhat smaller than for electron ionization. The specificity is dependent on the ionization conditions used and the uniqueness of the ions monitored. The ions produced from chemical ionization can be acquired in either the scan or SIM mode.

Acceptance criteria for compound identification with chemical ionization must be documented. The ion derived from the intact molecule [e.g., $(M+H)^+$ or $(M-H)^-$] or an ion closely related to the molecular species (e.g., loss of HF) should be monitored. Ion ratios compared to a standard run in the same batch should be within $\pm 25\%$ variation (e.g., for 80% relative abundance, acceptance range is 60 to 100%). Computation and evaluation of the ion ratios should be performed as described in Section 8.2.3. If full-scan acquisition is used, there should be no major ions present in the scan that are not derived from the proposed analyte.

8.2.5 Tandem Mass Spectrometry (MS/MS)

Tandem mass spectrometric methods may employ either electron or chemical ionization techniques. The fragmentation process is a physicochemical interaction, and as a result the ion intensity reproducibility is lower than that observed for electron ionization. The selective fragmentation of an ion of defined mass significantly increases the specificity of the technique. The ions produced from collision-induced fragmentation can be acquired in either the scan or SIM mode.

Acceptance criteria for compound identification with MS/MS must be documented. Collision conditions should be selected to ensure that the precursor ion is present in MS/MS scan. When monitoring one precursor to get one product ion, the resolution for the first mass analyzer should be set to unity (i.e. mass window equals one amu). If multiple ions are monitored for identification, ion ratios compared to a standard run in the same batch should be within $\pm 25\%$ variation (e.g., for 80% relative abundance, acceptance range is 60 to 100%). Computation and evaluation of the ion ratios should be performed as described in Section 8.2.3. If full-scan acquisition is used, there should be no major ions present in the scan that are not derived from the proposed analyte.

References

- ¹ Federal Register. 53 FR 11970. Mandatory Guidelines for Federal Workplace Drug Testing Programs: Final Guidelines Notice. 1988.
- ² Wu Chen NB, Cody JT, Garriott JC, Foltz RL, Peat MA, Schaffer MI. Report of 1988 Ad Hoc Committee on Forensic GC/MS: Recommended Guidelines for Forensic GC/MS Procedures in Toxicology Laboratories Associated with Offices of Medical Examiner and/or Coroners. *J Forensic Sci.* 1990;35:236-242.
- ³ Sphon JA. Use of mass spectrometry for confirmation of animal drug residues. *J Assoc Off Anal Chem.* 1978;61:1247-1252.
- ⁴ Baldwin R, Bethem RA, Boyd RK, et al. Limits to confirmation, quantitation, and detection. *J Am Soc Mass Spectrom.* 1997;8:1180-1190.
- ⁵ de Ruig WG, Dijkstra G, Stephany RW. Chemometric criteria for assessing the certainty of qualitative analytical methods. *Anal Chim Acta.* 1989;223:277-282.
- ⁶ Fetterolf DD, Yost RA. *Int J Mass Spectrom Ion Proc.* 1984;62(1);33-49.
- ⁷ *International Vocabulary of Basic and General Terms in Metrology.* 2nd ed. Geneva, Switzerland: International Organization for Standardization; 1993.
- ⁸ ISO/IEC Guide 2 General terms and their definitions concerning standardization and related activities. Geneva, Switzerland: International Organization for Standardization; 1996.
- ⁹ ISO/IEC Guide 25 General requirements for the competence of testing and calibration laboratories. Geneva, Switzerland: International Organization for Standardization; 1997.
- ¹⁰ IFCC/IUPAC: Approved recommendation (1978). Quantities and units in clinical chemistry. *Clin Chim Acta.* 1978;96:157F-183F.
- ¹¹ Price P. Standard definitions of terms relating to mass spectrometry: A report from the committee on measurements and standards of the American Society for Mass Spectrometry. *J Am Soc Mass Spectrom.* 1991;2:336-348.
- ¹² Duffus JH. Glossary for chemists of terms used in toxicology. *Pure and Appl Chem.* 1993;65:2003-2122.
- ¹³ Ettre LS. Nomenclature for chromatography. *Pure Appl Chem.* 1994;65:819-872.
- ¹⁴ David RB, Thompson JE, Pardue HL. Characteristics of statistical parameters used to interpret least square results. *Clin Chem.* 1978;24:611-620.
- ¹⁵ Kroll MH, Emmancipator K. A theoretical evaluation of linearity. *Clin Chem.* 1993;39:766-772.
- ¹⁶ Lipman HB, Astles JR. Estimating the linear analytic range. *Clin Chim Acta.* 199;282:15-34.
- ¹⁷ Currie LA. Nomenclature in evaluation of analytical methods, including detection and quantification capabilities (IUPAC Recommendations 1995). *Pure Appl Chem.* 1995;67:1699-1723.

- ¹⁸ Currie LA. Detection: Overview of historical, societal, and technical issues. In: *Detection in Analytical Chemistry: Importance, Theory, and Practice*. Washington, DC: American Chemical Society; 1988:1-62.
- ¹⁹ Hubaux A, Vos G. Decision and detection limits for linear calibration curves. *Anal Chem*. 1970; 42:849-855.
- ²⁰ Long GL, Winefordner JD. Limit of detection: A closer look at the IUPAC definition. *Anal Chem*. 1983;55:712A-724A.
- ²¹ Clayton CA, Hines JW, Elkins PD. Detection limits with specified assurance probabilities. *Anal Chem*. 1987;59:2506-2514.
- ²² Glaser JA, Foerst DL, McKee GS, Quave SA, Budde WL. Trace analysis for wastewaters. *Environ Sci Technol*. 1981;15:1426-1435.
- ²³ Lambert D, Peterson B, Terpenning I. Nondetects, detection limits, and the probability of detection. *J Am Stat Assoc*. 1991;266-277.
- ²⁴ Armbruster D, Tillman MD, Hubbs LM. Limit of detection (LOD)/limit of quantitation (LOQ): Comparison of the empirical and statistical methods exemplified with GC-MS assays of abused drugs. *Clin Chem*. 1994;40:1233-1238.
- ²⁵ Cleij P, Dijkstra A. Information theory applied to qualitative analysis. *Fr J Anal Chem*. 1979;298:97-109.
- ²⁶ Thieme D, Müller RK. Information theory and systematic toxicological analysis in "general unknown" poisoning cases. *Fr J Anal Chem*. 1997;358:785-792.
- ²⁷ Snyder LR, Kirkland JJ, Glajch JL. *Practical HPLC Method Development*. 2nd ed. New York: Wiley Interscience. 1997:714-720.
- ²⁸ de Ruig WG, Dijkstra G, Stephany RW. Chemometric criteria for assessing the certainty of qualitative analytical methods. *Anal Chim Acta*. 1989;223:277-282.
- ²⁹ Stein SE. An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectrometry data. *J Am Soc Mass Spectrom*. 1999;10:770-781.
- ³⁰ McLafferty FW, Hertel RH, Villwock RD. Probability based matching of mass spectra. *Org Mass Spectrom*. 1974;9:690-702.
- ³¹ Stein SE, Scott DR. Optimization and testing of mass spectral library searching algorithms for compound identification. *J Am Soc Mass Spectrom*. 1994;5:859-866.

Additional References

Boyd RK, Henion JD, Alexander M, et al. Mass spectrometry and good laboratory practice. *J Am Soc Mass Spectrom.* 1996;7:211-218.

Davis R, Frearson M, Prichard FE. *Analytical Chemistry by Open Learning, Mass Spectrometry.* New York: John Wiley & Sons; 1989.

Goldberger BA, Huestis MA, Wilkins DG. Commonly practiced quality control and quality assurance procedures for gas chromatography/mass spectrometry analysis in forensic urine drug-testing laboratories. *Forensic Sci Rev.* 1998;9:60-80.

International Conference on Harmonization. *ICH Harmonized Tripartite Guideline.* Adapted October 1994.

March RE, Todd JFJ, eds. *Practical Aspects of Ion Trap Mass Spectrometry.* Boca Raton: CRC Press. 1995:Vol. 1-3.

McLafferty FW, Turecek F. *Interpretation of Mass Spectra.* 4th ed. Mill Valley, CA: University Science Books; 1993.

Rood D. *A Practical Guide to the Care, Maintenance, and Troubleshooting of Capillary Gas Chromatography Systems.* Hüthig, Heidelberg; 1991.

Ullman MD, Bowers LD, Burtis CA. Chromatography/Mass Spectrometry. In: Burtis CA, Ashwood ER, eds. *Teitz Textbook of Clinical Chemistry.* 3rd ed. Philadelphia: Saunders. 1999:164-204.

NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

C43-P: Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Proposed Guideline

General

1. Consider including several figures that illustrate both a typical ion chromatograph and a total ion chromatograph.
 - **A figure has been placed at the end of Section 4.4.**
2. Overall I'd like to say the primary objective stated as to establish uniform practices for producing quality data for quantitation and identification of drug/metabolites was not met. To a practicing scientist, I don't believe that the explanations were specific enough. While most people have the impression that the SAMHSA guidelines are too strict and dictatorial, there is some comfort in knowing that interpretations are reproducible among labs following these criteria for their drug testing. When I read the title to this guideline, my first thought was great someone is finally going to set some standards in clinical and forensic medicine. However, I think specific examples for how to use these criteria in the clinical and forensic arenas are lacking.
 - **The SAMHSA guidelines are written in response to experience with the analysis of five compounds using primarily selected ion monitoring. It would be extremely difficult to cite examples for all of the possible scenarios for clinical and forensic testing. The commenter's opinion that the primary objective is not met, while appreciated, is not shared by the subcommittee.**

Foreword

3. Second paragraph, last sentence, "Appropriate application of these analytical tools requires that the methods are fit for their purpose and the instruments are operating correctly." This is somewhat vague. Are all the preceding methods mentioned in the context of being confirmatory methods? If so, perhaps the sentence should read 'Methods used are fit for the purpose of confirmation and....'
 - **The subcommittee believes that the sentence as written describes the situation accurately. Since the entire guideline applies only to confirmation analyses, the specifics of fit for purpose are determined by whether the compound must be quantified or identified.**

Introduction

4. In the Introduction, the statement "establish broader criteria" doesn't specifically say who the comparator is although it is inferred in the abstract that it is the SAMHSA guidelines.
 - **The wording has been changed to reflect that the criteria apply to areas other than drugs of abuse testing.**

Section 4

5. There are 5 pages of GC/MS instrument description. Scientists who are looking for guidelines involving GC/MS are probably quite familiar with the technique.
- **While this may be true, the experience of the subcommittee members is that significant amounts of incorrect information are circulated in the workplace. It is also likely that some scientists are not familiar with all of the techniques discussed. The subcommittee believes, therefore, that inclusion of this information establishes a uniform base upon which to build a guideline.**

Section 4.3.4

6. Section 4.3.4 touches on large amounts of air in the system. Section 7.2.2 helps identify ways to assess the system for air but nowhere is it included what instrument parts and parameters are damaged by air leaks.
- **There is specific reference to the damaging effects of air on the filament and column in Section 4.3.4. The deleterious effects on tuning are also indicated. The subcommittee therefore believes that this comment has been addressed.**

Section 6.1.1

7. For non-threshold compounds recommendation, should this include reference to whether these reference materials and metabolite isolates be run at the time of the unknown or run and stored as a database with which to compare the unknown spectrum? On the last line, it is unclear as to whether comparison of your unknown spectra or comparison of your reference materials to a reputable scientific journal is addressed here.
- **A sentence has been added to Section 6.1.1 to indicate that the secondary reference material should be analyzed contemporaneously with the unknown. It seems clear that the spectra obtained from the secondary reference material should be comparable to a spectrum published in the literature.**

Section 6.2

8. Second Paragraph: In this section on accuracy, it may be appropriate to reference CLIA '88 as one suggested interval for calibration verification to assess accuracy: according to CLIA it 'should be performed at least every 6 months and after major maintenance or change in reagents.'
- **A recommendation of at least every twelve months and after major maintenance has been made in Section 6.2.**

Section 6.2.1

9. Since carryover and contamination are the major sources of false positivity and inaccuracy in any high sensitivity measurements, the committee might consider strengthening Section 6.2.1 by including descriptions or examples of how to avoid or detect carryovers (e.g., negative runs...).
- **A sentence has been added to Section 6.2.1 using a blank injection as an example of a method to detect carryover.**

Section 6.4

10. Linearity, top paragraph. The discussion concerns choice of calibration concentrations for best precision about the regression line, rather than linearity per se. Perhaps a section should be added for calibration, or the heading for Section 6.4 could be changed to 'Calibration' with this paragraph moved first to precede discussion of linearity.
- **There is already a section on Calibration (Section 7.3). The subcommittee concludes that the inclusion of information on the assessment of linearity is best presented here.**
11. Linearity, first paragraph. If peak heights or areas (or ratios if an IS is used) are plotted as a function of analyte concentration to determine assay linearity, it should be clear that the approach to assess linearity must be the same as that used to obtain patient results.
- **Since the remainder of the section discusses the use of linear calibration to establish concentrations, this is implicit if not explicit in the discussion.**
12. Linearity, second paragraph. Correlation coefficient is a particularly poor way to assess assay linearity. It is not sensitive to deviations from linearity and the cut-off of 0.99 has no scientific basis. Reference should be made to NCCLS document EP6. For a discussion of the faults in using correlation coefficient, see Lipman and Astles, Clin Chim Acta, (1999) 282:15-34.
- **A revised paragraph discussing the limitations of correlation coefficient in determining linearity has been incorporated along with several additional references. A reference to NCCLS document EP6 was also added at the end of the section.**
13. Linearity, third paragraph. Authors may want to make the recommended frequency for checking linearity (currently yearly) to be the same as the frequency for checking calibration verification (Section 6.2).
- **The recommendation for linearity check has been changed to every twelve months.**

Section 6.5

14. It would be pertinent to include standard ways in which the industry is verifying LOD annually. Also, what specificity criteria should be applied to determining LOD for non-threshold compounds?
- **The third paragraph deals specifically with the current industry standard for verifying LOD.**

Section 6.6

15. It would be pertinent to include standard ways in which the industry is verifying LOQ annually.
- **The section deals specifically with the current industry standard for verifying LOQ.**

Section 7

16. Routine Instrument and Method Performance, second sentence. A statement is made that 'quality assurance samples' should be included with each batch. 'Quality control samples' would be more appropriate and that phrasing is used elsewhere (Section 7.4, first paragraph)."
- **The wording has been changed to "quality control samples."**

Section 7.1

17. Most people using GC do not even know what theoretical plates are let alone how to calculate them. It seems inappropriate to say they should calculate something we all know they probably will not. Perhaps something that they will actually do would be better.

The measurement of peak width at half height is something they can reasonably do. I think it should say something about ensuring the peak is represented in such a way that this measurement is possible. I have seen some peaks that are drawn on a wide time scale that the peak looks like nothing more than a spike, making the measurement impossible. Also, saying that time is acceptable to measure is reasonable, but distance seems inappropriate. Since the representation of a peak can be changed by telling the system how to draw it, as a result, distance can be satisfied by any peak. Measuring distance to determine asymmetry is reasonable. Perhaps I am not clear on what is meant by this paragraph.

- **Guidelines are intended to reflect good practice. If they can measure the peak width at half height, they can calculate theoretical plates.**

The purpose of measuring theoretical plates is twofold. First, since the width of the peak changes with the retention time (for an isothermal system), absolute width is not a good measure of anything. Second, since theoretical plates is a dimensionless ratio, the peak width and retention time can be measured in any units, so long as they are on the same scale (not expanding the scale for one and not the other). A definition of plate number has been included in Section 5 to assist in calculations.

18. Regarding the statement that "the rationale for recommendation of this resolution is the deviation is < 1% for...." Please reference this citation.

- **A reference has been added.**

Section 7.3

19. Section 7.3 doesn't address the differences in matrix found within the clinical and forensic practices.

- **A wide variety of matrices are found within clinical and forensic practice, and discussion of all of them is beyond the scope of this document.**

Section 7.4

20. Obtaining blind QC in these matrices presents the same problem. This also does not address how to make, how to store, and how to use these so-called quality control samples which should comprise 10% of an analytical batch.

- **The production of quality control materials is beyond the scope of this guideline. While the topic is of great interest, the strategies and limitations required for production of control materials in various matrices would require a separate document.**

Section 7.4.2

21. This might necessitate creation of a QC material with each confirmation run. While this practice is probably okay, there should be documentation that at some point the material used to create this QC or metabolite isolate was verified as to purity, etc. This criterion also suggests concentration matching

of a nonthreshold compound for which quantitation criteria have not been established and with no suggestion for how one might concentration match.

- **The production of quality control materials is beyond the scope of this guideline. While the topic is of great interest, the strategies and limitations required for production of control materials in various matrices would require a separate document.**

Section 7.4

22. It is not clear to me how a conjugated internal standard can be used to assess hydrolysis of a sample. This should be explained or I think it is something that people will not get right.

- **The subcommittee agrees and the last sentence of Section 7.4 has been deleted.**

Section 8.1

23. Most drug labs currently use $\pm 2\%$ for time. Is there any reason to set this to 1%? I have no real objection except we need to realize that this would make many labs change criteria. I do have serious concerns about using absolute retention time for differences. ± 0.2 minutes seems a problem for some long runs and unreasonably long for short retention time, particularly with the movement to shorter run times. As some drug testing labs work to get drug retention times down to 1-2 minutes, that criterion translates to $\pm 10\text{-}20\%$.

- **The purpose of this guideline is to address a broad spectrum of confirmation activities, not just drugs of abuse testing which is a high volume subset of the testing world. It would be appropriate to keep this broader spectrum of use in mind when discussing the guideline.**

That said, this is certainly a difficult issue. A $\pm 2\%$ interval at 20-minute retention time is ± 0.4 minutes; a 1% interval is ± 0.2 minutes which should be achievable. The subcommittee agreed that it should be possible to achieve 1% reproducibility in retention time. The phrase "whichever is smaller" was added to the first paragraph.

24. A valley of 20% is too high. Typically 10% is the most allowed. Why 20%? Also, there appears to be a discrepancy between this section and section 7.1. Also, it defines it as a valley assuming equal sized peaks, which seldom happens. There should be something said about unequal sized peaks, particularly when assessing the valley based on the peak of interest, not the largest (which many labs want to do).

- **The guideline now states that a resolution of 1.25 is required, which by definition is a 10% valley for equal sized peaks. The valley for a resolution of 1.25 changes with relative peak size, and for whether the peak of interest is the larger or the smaller peak. The point is that the use of a valley calculation is not recommended due to the changing situation with relative peak height. Additional discussion of the resolution issue is included in Section 7.1.**

Section 8.2.2

25. This section should give some guidelines about subtraction of background; not necessarily how to, but at least some things that are not appropriate.

- **The subcommittee believes this is beyond the scope of the document because there is not a standard approach which is varied between software programs.**

Section 8.2.3

26. At the end of the second to last paragraph discussion of not using different standards or different methods of computation for different specimens in a batch is discussed. While this is ideal, in the interest of time and cost, more than one presumptive positive may be included in a confirmation run. The article already discusses ways in which the concentration of a compound affects its identification and quantitation and adjustments that can be made to relative abundance and abundance criteria. I will say from experience that this is almost the rule and not the exception that specimens of differing concentrations, differing metabolites and differing ways in which individual bodies produce metabolites will be in the same batch confirmation run and will need essentially different identification criteria placed on them to determine positivity.

- **The comment, while relevant, does not indicate what the author would like the subcommittee to do.**

27. I get concerned with using absolute ion ratio ranges, particularly at the low end. Although I understand the problem with low abundance results, although my experience says it has more to do with low abundance than low relative abundance if the absolute abundance is high enough. No excuse for absolute ranges for urine drug testing except for a few compounds that are monitored at very low concentrations. Also paragraphs two and three give different suggestions of implementation. The first is particularly troublesome as I have inspected a lab that used absolute ranges that went from 0-20% for one of the PCP ions and guess what? 1% is inadequate in my view because that could well be background noise. To use such a criteria should be reserved only for rare and unique circumstances and not proposed as an option for most analyses.

- **The purpose of this guideline is to address a broad spectrum of confirmation activities, not just drugs of abuse testing, which is a high volume subset of the testing world. It would be appropriate to keep this broader spectrum of use in mind when discussing the guideline.**

This comment is somewhat difficult to understand, since by definition the mass spectrum is reported in relative abundance; thus the term “absolute abundance” is not clear.

Outside of the world of drugs of abuse, there are many examples of compounds that yield relatively few ions of relative abundance greater than 10%. Several publications have shown that in this case, the reproducibility of the ion signal from a mass spectrometer may not achieve $\pm 10\%$ — in essence, you are requiring better ion signal reproducibility for low intensity ions than you are for more intense ions.

The concept of using “0%” abundance for any ion makes little sense. This issue has been addressed in the third paragraph of Section 8.2.3.

Related NCCLS Publications*

- EP6-P2** **Evaluation of the Linearity of Quantitative Analytical Methods: A Statistical Approach; Proposed Guideline—Second Edition (2001).** This document provides guidelines for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.
- M29-A2** **Protection of Laboratory Workers from Occupationally Acquired Infections – Second Edition; Approved Guideline (2001).** This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of bloodborne infection from laboratory instruments and materials; and recommendations for the management of bloodborne exposure.
- NRSCL8-A** **Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).
- T/DM6-A** **Blood Alcohol Testing in the Clinical Laboratory; Approved Guideline (1997).** This document provides technical and administrative guidance on laboratory procedures related to blood alcohol testing, including specimen collection, methods of analysis, quality assurance, and reporting of results.
- T/DM8-A** **Urine Drug Testing in the Clinical Laboratory; Approved Guideline (1999).** This guideline addresses the development of procedures for analysis of urine to determine the presence of certain controlled substances; for specimen collection and processing; for methods of analysis; for quality assurance; and for the reporting and interpretation of results.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

NOTES

NCCLS ▼ 940 West Valley Road ▼ Suite 1400 ▼ Wayne, PA 19087 ▼ USA ▼ PHONE 610.688.0100
FAX 610.688.0700 ▼ E-MAIL: exoffice@nccls.org ▼ WEBSITE: www.nccls.org ▼ ISBN 1-56238-473-2





VIA OVERNIGHT MAIL and EMAIL

June 30, 2006

Joseph M. Papp
P. O. Box 329
Glen Spey, NY 12737
Joe.papp@mikefrayssesports.com

Re: Sample #941068 – International 42nd Presidential Cycling Tour of Turkey

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

Dear Mr. Papp:

Your urine sample collected at the International 42nd Presidential Cycling Tour of Turkey on May 7, 2006, was sent to the WADA accredited laboratory at Ankara, Turkey ("the Laboratory") for analysis. The Laboratory has reported that your A sample contains metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone), which are listed as prohibited substances in the class of anabolic androgenic steroids on the World Anti-Doping Agency's Prohibited List, adopted by both the USADA Protocol for Olympic Movement Testing ("Protocol") and the Union Cycliste Internationale ("UCI") Anti-Doping Rules. The Laboratory's positive A Sample report is enclosed with this letter. On June 27, 2006, USA Cycling requested USADA handle your positive case under the USADA Protocol.

At this time, in order to avoid delay in the adjudication of your case, you have the right to accept the Laboratory results. If you choose to accept the A Sample Laboratory results and do not wish to contest the finding of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone) in your sample, please sign the attached Acceptance of Laboratory Findings and Waiver Form and fax it to me at 719-785-2028 no later than July 10, 2006. Further, if you choose to accept the Laboratory results, we will forward your case immediately to a panel of the independent Anti-Doping Review Board, as set forth in the USADA Protocol, for its consideration. Under the USADA Protocol and the UCI Anti-Doping Rules, the finding of a prohibited substance or method in an athlete's sample constitutes a doping violation. If it is ultimately determined that this is your first doping violation, a sanction may be imposed that will include disqualification of any of your competitive results achieved from May 7, 2006, the day your sample was collected, and a two year period of ineligibility.

United States Anti-Doping Agency

1330 Quail Lake Loop, Suite 260, Colorado Springs, CO 80906 ■ Tel: 719.785.2000 ■ Fax: 719.785.2001

usada@usantidoping.org ■ www.usantidoping.org

GDC01359.1

If you choose not to accept the A Sample Laboratory results, your B Sample will be opened and analyzed at the Laboratory in Ankara, Turkey, on July 17, 2006. You and/or your representative have the right to be present at your expense to observe the B Sample opening and the analysis (which usually takes many hours in its entirety over multiple days). Please inform me in writing by fax at 719-785-2028 by July 10, 2006, if you plan to attend the B Sample analysis so that we may provide you with information on the time and address of the laboratory. Please contact me if you have any questions about the timing of the analysis. If you intend to compete in any protected competitions, USADA has the right under its Protocol, Section 13, to expedite this matter to final resolution prior to the protected competition.

Additionally, you have the right, at this time, to accept a "provisional suspension." By accepting a "provisional suspension," you will be immediately suspended from competing in all competitions under the jurisdiction of UCI, USA Cycling, and the United States Olympic Committee ("USOC"), until your case is deemed not to be a doping offense, you accept a sanction, you fail to contest this matter, or a hearing has been held in this matter. If you choose to accept this "provisional suspension," the time served under the "provisional suspension" will be deducted from any period of ineligibility that you might receive beginning on the date you accept the "provisional suspension" and notify USADA of such acceptance. If you choose not to accept this "provisional suspension," any period of ineligibility you might receive will begin on the date of your acceptance of the sanction or on the date of the arbitration hearing panel's decision.

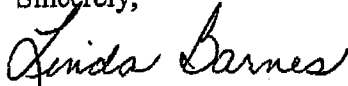
If you accept the "provisional suspension," USADA will give notice to the USOC, UCI, and USA Cycling of your acceptance of the "provisional suspension." Your decision to accept this "provisional suspension" is purely optional. You do not have to accept this "provisional suspension" in order to proceed with your case. **If you are willing to accept a "provisional suspension," please inform us in writing by July 10, 2006, by executing and returning the attached USADA Acceptance of Provisional Suspension Form.**

Also of importance, you are still subject to testing pending the outcome of this matter.

USADA will not publicly disclose or comment on the specifics of your test results until your case has been resolved. By copy of this letter, USADA is notifying USA Cycling and the USOC of your test results and requests that these organizations not comment publicly concerning this information until disclosed as provided in the USADA Protocol.

Enclosed for your reference are copies of the USADA Protocol and the World Anti-Doping Code, which set forth the administrative procedures followed for positive or elevated test results. You may also wish to contact John Ruger, the USOC Athlete Ombudsman who is completely independent of USADA, or your own personal attorney, for assistance or further information. Mr. Ruger may be reached at One Olympic Plaza, Colorado Springs, CO, 80909, by telephone at (888)-ATHLETE, by fax at (303) 444-6626 or by e-mail at John.Ruger@usoc.org, or at www.888athlete.org.

Sincerely,



Linda M. Barnes
Testing Results Manager

cc: Sean Petty, USA Cycling (w/o encls.)
Gary Johansen, USOC Deputy General Counsel (w/o encls.)
Jim Scherr, USOC (w/o encls.)

Enclosures: UCI Anti-Doping Control Test Certificate
Laboratory Certificate of Analysis
UCI Anti-Doping Rules
World Anti-Doping Code
WADA List of Prohibited Substances
USADA Protocol
USOC Anti-Doping Policies

UNITED STATES ANTI-DOPING AGENCY
ACCEPTANCE OF PROVISIONAL SUSPENSION

I, Joseph M. Papp, accept a "provisional suspension" as a result of the finding of the prohibited metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone) in my urine Sample #941068, collected at the International 42nd Presidential Cycling Tour of Turkey on May 7, 2006.

I understand and accept that I will not be able to compete in any competitions under the jurisdiction of the Union Cycliste Internationale ("UCI"), USA Cycling, or the United States Olympic Committee ("USOC") while serving this "provisional suspension."

I understand that the period of the "provisional suspension," beginning on the date I accept this "provisional suspension" and notify USADA of such, will be deducted from any period of ineligibility that I might receive in my case.

I understand and accept that USADA will notify UCI, USA Cycling, and the USOC of my acceptance of the "provisional suspension."

I understand and accept that my acceptance of the "provisional suspension" is purely voluntary and optional. I understand and accept that I am entitled to proceed with my case, to a hearing if necessary, regardless of whether I accept this "provisional suspension."

I understand and accept that I may serve this "provisional suspension" and it may ultimately be determined that no doping offense has occurred by the Panel of the USADA Anti-Doping Review Board or through a hearing.

I understand and accept that I am still subject to testing pending the outcome of this matter.

Signature of Joseph M. Papp

Date

Printed Name of Joseph M. Papp

UNITED STATES ANTI-DOPING AGENCY

ACCEPTANCE OF LABORATORY FINDINGS
WAIVER OF RIGHT TO B SAMPLE ANALYSIS AND
WAIVER OF RIGHT TO CONTEST LABORATORY FINDINGS

I, Joseph M. Papp, accept the finding of the WADA accredited laboratory at Ankara, Turkey (the "Laboratory") that my urine Sample #941068 collected on May 7, 2006 at the International 42nd Presidential Cycling Tour of Turkey contains the prohibited metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone). I understand that the Laboratory's findings are based on analysis of the A Sample of urine Sample #941068 and that the B Sample analysis on this Sample has not been conducted. I further understand that pursuant to the Protocol for Olympic Movement Testing of the United States Anti-Doping Agency, a sample shall not be considered positive until after the B Sample analysis confirms the A Sample analysis or the athlete has expressly waived the B Sample analysis. A waiver of the B Sample analysis means the sample shall be considered positive pursuant to the findings of the A Sample analysis. I have been advised of my right to have the B Sample analysis conducted and I voluntarily, knowingly, and intelligently waive my right to have a B Sample analysis conducted on Sample #941068. I do not contest the Laboratory's finding that metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone) were in my urine sample. I voluntarily, knowingly, and intelligently accept the Laboratory findings and waive any right to contest the results of the Laboratory with respect to my Sample collected on May 7, 2006.

Signature of Joseph M. Papp

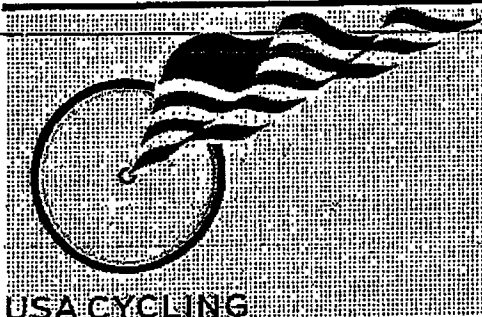
Printed Name of Joseph M. Papp

Date

Summary by USADA
Of Laboratory Documents
For Sample #941068

| | |
|-----------------------------|---|
| Sport: | Cycling |
| Sample Collection Date: | May 7, 2006 |
| Type of Collection: | In-Competition International 42 nd Presidential Cycling Tour of Turkey |
| WADA Accredited Laboratory: | Ankara, Turkey |
| Substance: | Metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH- androsterone) |

This summary is based on the laboratory documents provided and is not intended to replace, substitute or in anyway supersede the laboratory documents. This summary is only for general reference purposes.



USA CYCLING

1 Olympic Plaza, Colorado Springs, CO 80909-5775

Facsimile Cover Sheet

To: Travis Tygart
Company: USADA
Phone:
Fax: 785-2028

From: Sean Petty
Company: USA Cycling
Phone: 719-866-4783
Fax: 719-866-4596

Date: June 26, 2006

**Pages including this
cover page:** 7

Comments:

Travis,
Attached is what we received from the UCI on File 22/06.

All the best,
Sean

26.JUN.2006 11:03

N0310 — P. 1 —



UNION CYCLISTE INTERNATIONALE

CH 1860 Aigle / Suisse

Tél. : +41 24 468 58 11 - Fax : +41 24 468 58 68 - e-mail : christian.varin@uci.ch

FAX MESSAGE

To : USA CYCLING
Mr. Steve JOHNSON
Fax nbr. : +1 719 866 46 28
From : Christian Varin, Manager
Copy : Agence Mondiale Antidopage
Mme Janie Soublière
+1 514 904 45 45
USADA
Mr. Tygart
+1 719 785 20 01
Date : June 26th 2006
Ref : Anti-Doping Services/ Cvd/DI
Total pages : 6 (including this one)
Subject : File 22/06

URGENT
CONFIDENTIEL

This facsimile may contain information that is confidential and which may be subject to legal privilege. If you are not the intended recipient, you must not pursue, use, disseminate or copy this message. If you received this message in error, please notify us by telephone (+41 24.468.58.11) and return the original message by mail. Thank you.

AVERTISSEMENT : Le contenu du présent fax ainsi que les documents qui y sont joints sont protégés par le secret professionnel. Toute communication, copie ou révélation de leur contenu à une personne autre que leur destinataire est strictement interdite et pénalement sanctionnée. Au cas où ce fax ne vous serait pas destiné nous vous remercions de bien vouloir nous en aviser immédiatement par téléphone (+41.24.468.58.11) et nous retourner l'original par courrier.

26.JUN.2006 11:03

N0310

P.2



INTERNATIONAL CYCLING UNION

Registered / Confidential

USA CYCLING

Mr. Steve JOHNSON

One Olympic Plaza

CO-80909 COLORADO SPRINGS

USA

Aigle, July 20th 2005

Ref: Antidoping / Cv / DI

File Nr. 22/06 (to mention in your correspondence)Joseph PAPP (licence 0027194 - UCI Code USA19750525) / Tour of Turkey (TUR), 7th May 2006

Dear Sir,

We inform you that the rider Joseph PAPP tested positive (OH-androstenedione, OH-androsterone) at the above-mentioned race according to the report we received from the Laboratory of Ankara (TUR), a copy of which we enclose (pursuant to article 187 of the Anti-Doping Rules). According to the laboratory's result on the A sample, the Anti-doping Commission must start from this notion that an offence against the mentioned Regulations has objectively been committed.

The rider is allowed to require without delay the B sample analysis (in accordance with articles 191 and following of the Anti-Doping Rules), or, failing that, it will be considered that he has renounced to this right. Mr. Joseph PAPP and/or his representative have also the right to attend the opening of the B sample and his analysis if this one is required. The rider is also allowed to require a copy of the complete analysis report for the samples A and B. In accordance with article 194 AR, to be acceptable the request for the analysis of the B sample shall be sent by the national federation no more than 5 working days after receipt of the letter informing it of the adverse analytical finding.

Please be reminded that, according to the Anti-doping Regulations - chapter X, the final sanction is foreseen by art. 261 and reads as follows:

Art. 261***Imposition of Ineligibility for Prohibited Substances and Prohibited Methods***

Except for the specified substances identified in article 262, the period of ineligibility imposed for a violation of article 15.1 (presence of Prohibited Substance or its Metabolites or Markers), article 15.2 (Use or Attempted Use of Prohibited Substance or Prohibited Method) and article 15.6 (Possession of Prohibited Substances and Methods) shall be:

26.JUN.2006 11:03

N0310

P.3



First violation: 2 (two) years' Ineligibility

Second violation: lifetime ineligibility

However, the License-Holder shall have the opportunity in each case, before a period of Ineligibility is imposed, to establish the basis for eliminating or reducing this sanction as provided in articles 264 and 265.

We would like to remind you that a violation of these Anti-Doping Rules in connection with an In-Competition test automatically leads to Disqualification of the individual result obtained in that Competition according to article 256. In addition, we also ask you to take into consideration the articles 257 to 260.

Regarding the cost of the proceedings, please refer to articles 244 to 246.

Moreover, we communicate you the text of the article 9.2.002 of the UCI Regulations which provides:

A rider against whom an investigation was opened in relation to a fact which may cause a breach of the UCI Anti-Doping Rules, will not be eligible for the World Championships until the end of the suspension or until his definitive acquittal. In the event of a positive A sample, this clause applies starting from the notification of the abnormal analysis result to the rider.

Unless otherwise decided by the anti-doping commission, the above paragraph is also applicable in the event of an investigation or a procedure regarding such a fact, opened in pursuance of a law or other regulation.

Specific cases are examined by the anti-doping commission or its president. Their decision is without appeal.

In addition to the disqualification, the licensee and his national federation will be respectively sanctioned by a fine of CHF 2000 to CHF 10000.

The present condition for participation, aims to protect the integrity, serenity and reputation of the World Championships. Its application does not prejudice the decision whether an anti-doping violation has occurred and shall not give rise to any claim in the events of acquittal.

We would consequently ask you to implement proceedings according to articles 188 to 205 of those Regulations. We would remind you that, according to the articles 249 to 251, you have to keep us informed of all measures you take by sending us copies thereof.

Looking forward to hearing from you.

Sincerely yours,

Antidoping Services,

Christian VARIN, Manager

Enc: mentioned

Copy to: WADA
USADA



26.JUN.2006 11:03

TURKISH DOPING CONTROL

03123052062 03123052262

N0310

P.4

HAZ. 13 2006 16:20 S1

KİŞİYE ÖZ
GİZLİTÜRKİYE
DOPING
KONTROL
MERKEZİ

Sayı: B.30.2.HAC.0.AP.00.00/2006-472

Türkiye Doping Kontrol Merkezi / Turkish Doping Control Center

29.05.2006

Analiz Sertifikası / Certificate of Analysis

Gençlik ve Spor Genel Müdürlüğüne,
Ulus/AnkaraCc: 1- WADA / Janis Soublière
2- BADNAG Başkanlığı
3- UCI

| No | Konu / Subject | Açıklama / Explanation |
|----|--|--|
| 1 | Federasyon-Spor / Federation-Sport | Bisiklet/ Cycling |
| 2 | Organizasyon / Event | Uluslararası 42. Cumhurbaşkanlığı Türkiye Bisiklet Turu / International 42th Presidential Cycling Tour of Turkey |
| 3 | Yarışma-Yarışma dışı / In Competition-Out of Competition | Yarışma / In competition |
| 4 | Sporcunun cinsiyeti / The gender of the athletes | 1 Erkek / 1 Male |
| 5 | Numunenin alınış tarihi ve yeri / Date and place of sample collected | 05-07.05.2006 - Antalya-Finike |
| 6 | Numunenin Merkeze geliş tarihi / Date of sample accepted | 09.05.2006 |
| 7 | Analize başlama ve bitiş tarihleri / Date of start and end of the analysis | 11.05.2006 / 25.05.2006 |
| 8 | Numunenin tipi (Kan/İdrar) / Type of the samples (Urine/Blood) | İdrar / Urine |
| 9 | Doping kontrol numune sayısı / Number of the samples | 1 (10 numunelik grubun içinden) / 1 (from a group of 10 samples) |
| 10 | Kayıt Numarası / Reception Batch | D2006RECE190 |

GİZLİ EVRAKTIR. İzinsiz paylaşılmaz.

1/2

Hacettepe Üniversitesi, 08100 Sıhhiye, Ankara
Tel: 0 312 810 67 76 Faks: 0 312 305 20 62 e-posta: tdcm@hacettepe.edu.tr

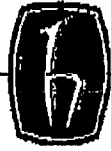
HEURE DE RECEPTION 13. JUN. 15:09

HEURE D'IMPRESSION 13. JUN. 15:10

GDC01359.11

26.JUN.2006 11:03
FROM : TURKISH DOPING CONTROL

03123052062 03123052062

N2310 P.5
HFZ. 13 2006 16:21 S2TÜRKİYE OZE!
GİZLİTÜRKİYE
DOPING
KONTROL
MERKEZİ

| Lab. Kodu (A) / Lab. Code (A) | Şişe Kod Numarası (A) / Bottle Code (A) | WADA listesine göre numune de bulunan yasaklı madde ve/veya metaboliti ile kullanılan yasaklı yöntem / Prohibited substances and/or their metabolites according to WADA found in urine | Bildirilmesi gereken madde derişimi / Urinary concentrations that must be reported |
|-------------------------------------|---|--|--|
| D0601342 | 941068 | Metabolites of testosterone or precursors (Testosteron veya öncüsü metabolitleri) 6 α -OH-androstenedione 6 β -OH-androsterone | - |

Türkiye Doping Kontrol Merkezi'ne uygun koşullarda gelen yukarıdaki idrar numunesi(leri)nde, PROC1A, PROC2A, PROC4A, PROC4B, PROC5A, PROC6A ve PROC6B metotları kullanılarak gaz kromatografisi-kitle spektrometrisi ve immünoassay yöntemleri ile uyarıcı, narkotik, beta-blokör, anabolik ajan, kannabis metaboliti, diüretik, kokain metaboliti ve hCG grubu ilaçların tarama analizleri gerçekleştirilmiştir. Bu analizlerin sonucunda, yukarıdaki şişe kod numaralı sporcunun idrarında, Dünya Dopingile Mücadele Kurulu (WADA) tarafından yayınlanan "Dünya Dopingile Mücadele Yönetmeliği 2005 Yılı Yasaklı Maddeler Listesi Uluslararası Standardı" listesinde, yukarıda adı geçen yasaklı madde bulunmuştur.

Yukarıda adı geçen maddenin doğrulama analizi, gaz kromatografisi-kitle spektrometrisi ile gerçekleştirilmiştir.

The urine sample(s) received in good order above was (were) screened for stimulants, narcotics, beta-blockers, anabolic agents, metabolite of cannabis, diuretics, metabolite of cocaine and hCG with the methods PROC1A, PROC2A, PROC4A, PROC4B, PROC5A, PROC6A and PROC6B by using gas chromatography-mass spectrometry and immunoassay in Turkish Doping Control Center. After the analyses the substance above from the list of prohibited agents and methods issued by World Anti-Doping Agency (WADA) have been found.

The presence of the substance was confirmed in the sample by using gas chromatography-mass spectrometry.

Not: Pozitif numune sonucuna itiraz olduğu takdirde, bu rapor tarihinden (29.05.2006) itibaren en geç 3 hafta içerisinde merkezimiz ile bağlantı kurularak analiz için uygun bir tarih belirlenmeli ve B numunesi analizi en geç 29.06.2006 tarihinde bitirilerek rapor edilmelidir. WADA kuralları gereği, bu tarih geçtikten sonra itirazda bulunulmuş olsun ya da olmasın, B numunesi analizi yapılmayacaktır.

Prof. Dr. M. Aytekin Temizer
WADA and ISO 17025 UKAS No: 2436 Accredited
Director of Turkish Doping Control Center

2/2

Hacettepe Üniversitesi, 06100 Sıhhiye, Ankara
Tel: 0 312 310 57 78 Faks: 0 312 305 20 52 e-posta: tdcm@hacettepe.edu.tr

HEURE DE RECEPTION 13. JUN. 15:09

HEURE D'IMPRESSION 13. JUN. 15:10

GDC01359.12

İzinsiz çoğaltılamaz.

GİZLİ DOKÜMAN.

26.JUN.2006 11:03 N0310 P.6
FROM : BSGM +90 312 3119637 HAZ. 20 2006 14:18 56



Union Cycliste Internationale



CONTRÔLE ANTIDOPAGE

ANTIDOPING CONTROL

Attestation du déroulement du contrôle

Test certificate

- Contrôle urinaire / Urine test ☒
1. En compétition / In competition ☒
Hors compétition / Out of competition ☐
3. Date: 08-05-2006
5. Course (nom, étape): International 42th Presidential
Race (name, stage): Cycling Tour of Turkey
7. Nom du coureur:
Surname of rider: PAPP

- Contrôle sanguin / Blood test ☐
2. Sexe: Masculin / Male ☒
Sex: Féminin / Female ☐
4. Lieu / Place: TURKEY - ALANYA
6. Discipline: ROAD
Discipline: ROAD
- Prénom: JOSEPH M
First name: JOSEPH M

Adresse du coureur:
Address of the rider: Via Hyacinthe Montebelli 26 51016 Montebelli Terme PT. Italia

9. Fédération nationale ayant délivré la licence:
National federation which delivered the license:

8. Code UCI:
UCI Code: USA 1975052-5

USA CYCLING FEDERATION

10. Numéro de licence:
License number: 0027194

11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☒

12. Heure de présentation:
Presentation time: 13:15
Volume 10.5 cc pH 6.5
Optional / Optional

13. Heure de prélèvement:
Time of sampling: 13:35
Densité / Specific gravity: 1.015

14. Code des flacons:
Bottle codes: 341068 A+B

15. Heure de refus du coureur:
Time of rider's refusal: 14:30

16. Médicaments pris:
Pharmaceutical drugs taken by rider: MILKATIL ALFAXIL NOVALGIN SINGULAIR HYDROCOUSINE

Contenu du livret de santé:
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT):
Therapeutic use exemption (TUE) Oui / Yes ☒ Non / No ☐

18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy: [Signature]

19. Accompagnateur:
Assistant: [Signature]

20. Médecin contrôleur:
Examining doctor: ENDEN Sizer

Agent de prélèvement sanguin:
Blood collection officer: [Signature]

21. Inspecteur antidopage:
Antidoping inspector: [Signature]

HEURE DE RECEPTION: 20. JUN. 14:08 HEURE D'IMPRESSION: 20. JUN. 14:13

GDC01359.13

HP LaserJet 4100 MFP



U.S. Anti-Doping Agency
7197852028
06/26/2006 05:06 PM

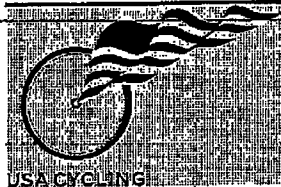
Fax Call Report

| Job | Date/Time | Type | Identification | Duration | Pages | Result |
|------|----------------|---------|----------------|----------|-------|--------|
| 1352 | 06/26 05:02 PM | Receive | 7198664596 | 03'50 | 7 | OK |

2006/TUE/26/MON 04:55 PM USA CYCLING

FAX No. 7198664596

P.001



1 Olympic Plaza, Colorado Springs, CO 80909-3775

Facsimile Cover Sheet

To: Travis Tygart
Company: USADA
Phone:
Fax: 785-2028

From: Sean Petty
Company: USA Cycling
Phone: 719-866-4783
Fax: 719-866-4596

Date: June 26, 2006
Pages including this
cover page: 7

Comments:

Travis,
Attached is what we received from the UCI on File 22/06.

All the best,
Sean

Catlin is leaving UCLA anti-doping lab

One of the world's leading authorities in sports doping, he is turning to full-time independent research.

By Michael A. Hiltzik, Times Staff Writer
March 14, 2007

Don H. Catlin, who founded UCLA's Olympic drug-testing laboratory in 1982 and built it into the busiest such facility in the world, said Tuesday he will retire from the university and turn to full-time research in sports doping.

"Basically, I know how to test," he said in a telephone interview from his Westside office, "but I don't have enough time anymore for my favorite avocation, which is research. Now I'll get to pick and choose my pet projects."

Catlin, 68, said those include developing a urine test for human growth hormone, which is reportedly growing in popularity as a sports doping substance, and improving the existing test for erythropoietin, or EPO, a performance-enhancing hormone that promotes the growth of red blood cells.

He will work through the Anti-Doping Research Institute, a non-profit lab he founded and that is located about a mile from the Olympic lab in West L.A. The institute, which is unaffiliated with UCLA, received a three-year, \$500,000 grant from Major League Baseball last year to develop an HGH test, which is currently undetectable in urine.

Catlin says the lab also has a commitment from the U.S. Anti-Doping Agency to provide it with a mass spectrometer, a crucial machine in drug testing, for a nominal lease fee, and is talking with the National Football League and other organizations to raise further funds.

"We're trying to get a handle on whether sport is really able and willing to support a research institute," he said.

As a professor at UCLA medical school, Catlin founded the Olympic lab at the request of the International Olympic Committee to provide drug testing at the 1984 Los Angeles Games. It is now part of a network of 34 anti-doping labs accredited by the World Anti-Doping Agency.

Of those, it is by far the busiest: Last year, the lab performed roughly 40,000 tests for anti-doping agencies, the NCAA, minor league baseball, and the NFL — nearly four times as many as the runner-up lab (in Cologne, Germany). The Olympic lab employs about 40 people.

One of the world's leading authorities in sports doping, Catlin's reputation is closely tied to the lab, and it is unclear whether the facility will retain its prominence after his departure. A spokesman for UCLA said the university hopes to keep the lab operating as part of its David Geffen School of Medicine. In a statement issued Tuesday, the Geffen school's dean, Gerald S. Levey, said he would "immediately begin discussions" with WADA and the other lab clients "with respect to the continuation" of the lab's association with the school.

Catlin said the transition to a new lab director, yet to be selected, would take about a month, and that he would become an emeritus professor at the Geffen school, where he plans to continue teaching.

As lab director, Catlin notched numerous milestones in the field of sports doping, notably his identification in 2003 of tetrahydrogestrinone (THG) a "designer steroid" illicitly dispensed to athletes by the notorious Bay Area Laboratory Cooperative, or BALCO.

But he has also been critical of the existing anti-doping system, which he believes is underfunded by sports and anti-doping organizations and unduly fixated on proving athletes dirty. Instead, he has proposed giving athletes an incentive to establish their cleanliness by volunteering for long-term medical profiling.

Under WADA rules, Catlin has been barred from offering his expertise as a witness to athletes defending themselves against anti-doping charges. He said Tuesday that he was unsure under what circumstances he would take on such assignments as an independent researcher.

"I'm not going to be out there testifying against my old friends," he said. "But I'll be out there trying to get it right for sport, for the agencies, for the athletes. It has to be right for everybody."

Catlin is leaving drug-testing lab at UCLA

One of the world's leading authorities in sports doping, he is turning to full-time independent research.

By MICHAEL A. HILTZIK
Times Staff Writer

Don H. Catlin, who founded UCLA's Olympic drug-testing laboratory in 1982 and built it into the busiest such facility in the world, said Tuesday he will retire from the university and turn to full-time research in sports doping.

"Basically, I know how to test," he said in a telephone interview from his Westside office, "but I don't have enough time anymore for my favorite avocation, which is research. Now I'll get to pick and choose my pet projects."

Catlin, 68, said those include developing a urine test for human growth hormone, which is reportedly growing in popularity as a sports doping substance, and improving the existing test for erythropoietin, or EPO, a perform-

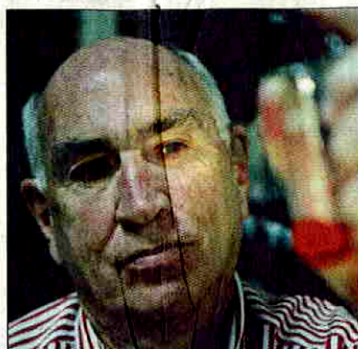
ance-enhancing hormone that promotes the growth of red blood cells.

He will work through the Anti-Doping Research Institute, a non-profit lab he founded and that is located about a mile from the Olympic lab in West L.A. The institute, which is unaffiliated with UCLA, received a three-year, \$500,000 grant from Major League Baseball last year to develop an HGH test, which is currently undetectable in urine.

Catlin says the lab also has a commitment from the U.S. Anti-Doping Agency to provide it with a mass spectrometer, a crucial machine in drug testing, for a nominal lease fee, and is talking with the National Football League and other organizations to raise further funds.

"We're trying to get a handle on whether sport is really able and willing to support a research institute," he said.

As a professor at UCLA medical school, Catlin founded the Olympic lab at the request of the International Olympic Committee to provide drug testing at the 1984 Los Angeles Games. It is now part of a network of 34 anti-



ALLEN J. SCHABEN Los Angeles Times

PIONEER: As director of the Olympic drug-testing lab at UCLA, Don H. Catlin notched numerous milestones in the field of sports doping.

doping labs accredited by the World Anti-Doping Agency.

Of those, it is by far the busiest: Last year, the lab performed roughly 40,000 tests for anti-doping agencies, the NCAA, minor league baseball, and the

NFL — nearly four times as many as the runner-up lab (in Cologne, Germany). The Olympic lab employs about 40 people.

One of the world's leading authorities in sports doping, Catlin's reputation is closely tied to the lab, and it is unclear whether the facility will retain its prominence after his departure. A spokesman for UCLA said the university hopes to keep the lab operating as part of its David Geffen School of Medicine. In a statement issued Tuesday, the Geffen school's dean, Gerald S. Levey, said he would "immediately begin discussions" with WADA and the other lab clients "with respect to the continuation" of the lab's association with the school.

Catlin said the transition to a new lab director, yet to be selected, would take about a month, and that he would become an emeritus professor at the Geffen school, where he plans to continue teaching.

As lab director, Catlin notched numerous milestones in the field of sports doping, notably his identification in

2003 of tetrahydrogestrinone (THG) a "designer steroid" illicitly dispensed to athletes by the notorious Bay Area Laboratory Cooperative, or BALCO.

But he has also been critical of the existing anti-doping system, which he believes is underfunded by sports and anti-doping organizations and unduly fixated on proving athletes dirty. Instead, he has proposed giving athletes an incentive to establish their cleanliness by volunteering for long-term medical profiling.

Under WADA rules, Catlin has been barred from offering his expertise as a witness to athletes defending themselves against anti-doping charges. He said Tuesday that he was unsure under what circumstances he would take on such assignments as an independent researcher.

"I'm not going to be out there testifying against my old friends," he said. "But I'll be out there trying to get it right for sport, for the agencies, for the athletes. It has to be right for everybody."

michael.hiltzik@latimes.com



CONFIDENTIAL

UCLA OLYMPIC ANALYTICAL LABORATORY
DEPARTMENT OF PHARMACOLOGY
UCLA SCHOOL OF MEDICINE
2122 GRANVILLE AVENUE
LOS ANGELES, CALIFORNIA 90025
PHONE: 310-825-2635
FAX: 310-206-9077

July 10, 2006

Terrence P. Madden
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906-4651

RE: Specimen number USADA [REDACTED], Site ID OOC = [REDACTED]

Dear Mr. Madden:

Please find enclosed the documentation package for the case identified above.

Enclosed are authentic photocopies of the original documentation supporting our conclusion and the drug testing report.

Please feel free to call if you have any questions.

Sincerely,

A handwritten signature in cursive script, appearing to read "Michael Sekera".

Michael Sekera
Certifying Scientist

cc: Don H. Catlin

CONFIDENTIAL
DOCUMENTATION

SAMPLE IDENTIFICATION:

Organization requesting test: USADA
Date of sample collection: May 28, 2006
Site ID: OOC
USADA Sample Code Number: [REDACTED]
UCLA Code: [REDACTED]
Substances identified: T/E ratio greater than four

TABLE OF CONTENTS

"A" SAMPLE CONFIRMATION DOCUMENTATION

Page

GC/IRMS Data

Negative urine QC

| | |
|--|---|
| Chromatogram of ion 44..... | 3 |
| Table of $\delta^{13}\text{C}$ values..... | 3 |

Positive calibrator (unextracted androsterone and etiocholanolone standards)

| | |
|--|---|
| Chromatogram of ion 44..... | 4 |
| Table of $\delta^{13}\text{C}$ values..... | 4 |

Sample urine aliquot

| | |
|--|---|
| Chromatogram of ion 44..... | 5 |
| Table of $\delta^{13}\text{C}$ values..... | 5 |

TMS derivative, SIM GC-MS data, IS = Ethylmorphine

Negative urine QC

| | |
|---|---|
| Extracted ion chromatogram of Testosterone, Epitestosterone and IS..... | 6 |
|---|---|

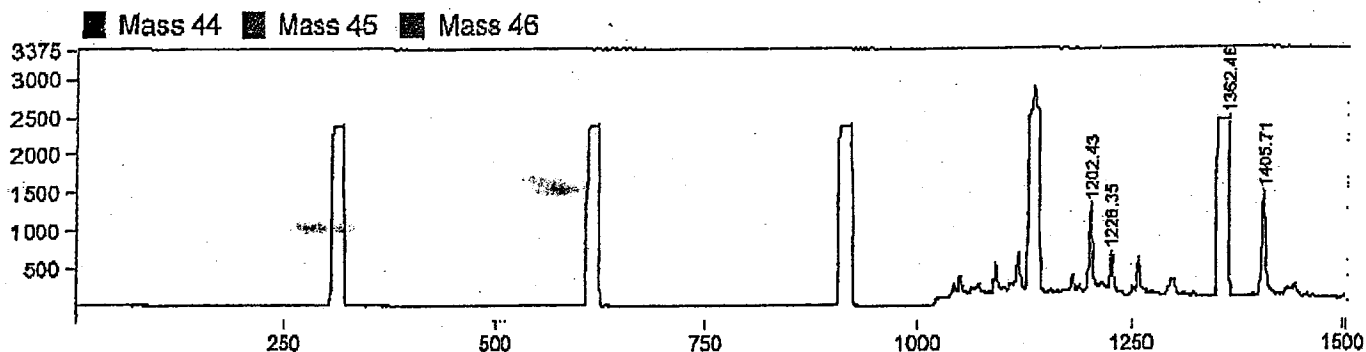
Positive calibrator (unextracted testosterone and epitestosterone standards at a ratio of 4:1)

| | |
|---|---|
| Extracted ion chromatogram of Testosterone, Epitestosterone and IS..... | 7 |
|---|---|

Sample urine aliquot

| | |
|---|---|
| Extracted ion chromatogram of Testosterone, Epitestosterone and IS..... | 8 |
|---|---|

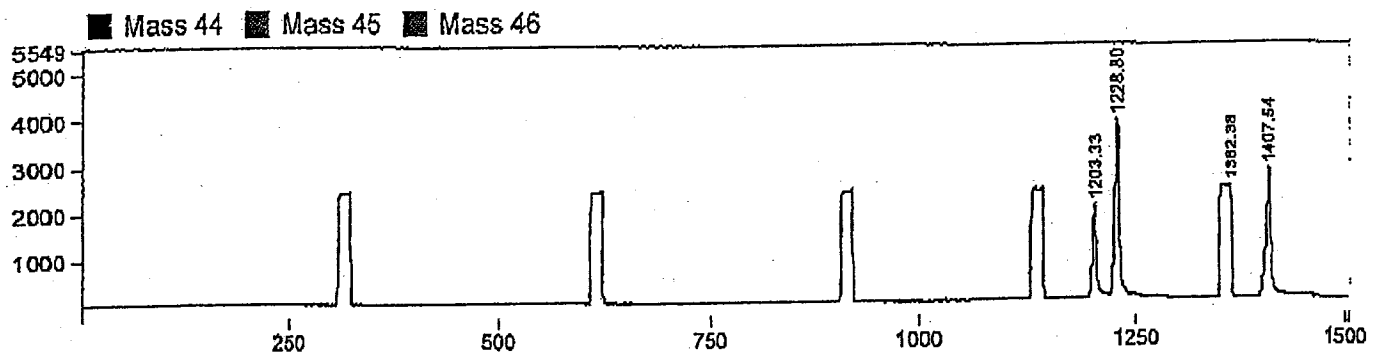
| | AS S | AS Method | Identifier 1 | Comment | Preparation | Post Script | Method |
|---|------|----------------|--------------|---------|-------------|-------------|--------------------|
| X | 48 | >Internal No 9 | NEGQC#5 | | | | method1[diois].met |



| Rt [s] | δ 13C/12C [per mil] vs. VPDB |
|--------|-------------------------------------|
| 1202.4 | -26.084 |
| 1226.3 | -27.427 |
| 1362.3 | -25.862 |
| 1405.7 | -25.862 |

BA 7/7/06

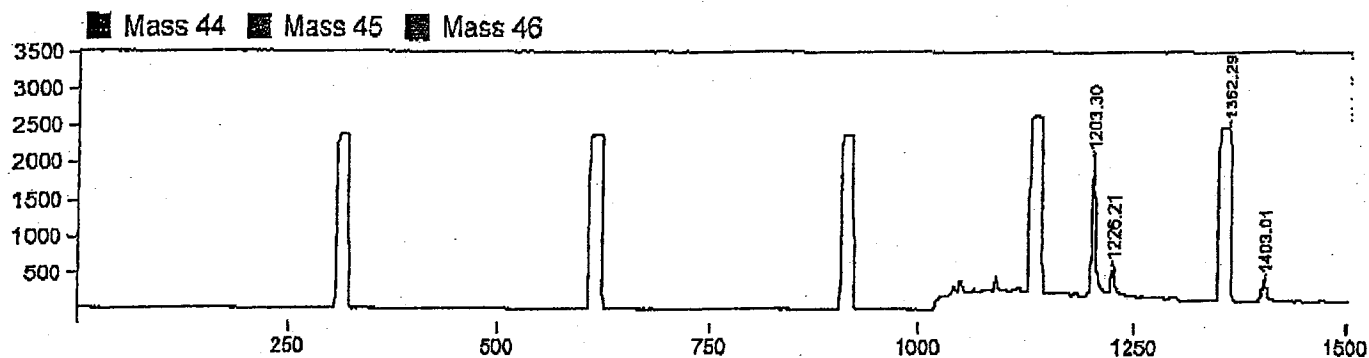
| AS S | AS Method | Identifier 1 | Comment | Preparation | Post Script | Method |
|------|-----------|----------------|----------|-------------|-------------|--------------------|
| X | 46 | >Internal No 9 | Diol STD | | | method1[diols].met |



| Rt [s] | d 13C/12C [per mil] vs. VPDB |
|--------|------------------------------|
| 1203.3 | -37.658 |
| 1228.8 | -36.640 |
| 1382.4 | -33.510 |
| 1407.5 | -23.130 |

BA 7/7/06

| AS | AS S | AS Method | Identifier 1 | Comment | Preparation | Post Script | Method |
|----|------|----------------|--------------|---------|-------------|-------------|--------------------|
| X | 52 | >Internal No 9 | | | 10 mL | | method1[diols].met |



| Rt [s] | d 13C/12C [per mil] vs. VPDB |
|-----------|------------------------------------|
| 1203.3 | -32.736 |
| 1226.2 | -31.737 |
| 1362.3 | -33.540 |
| 1403.0 | -25.417 |

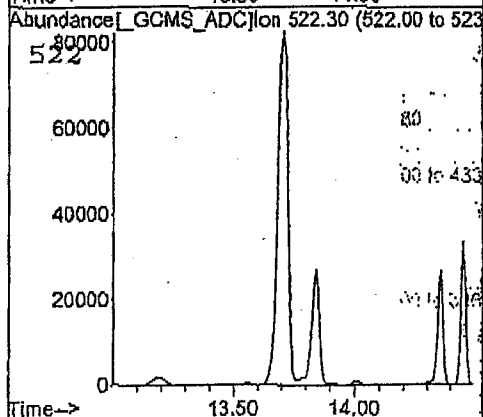
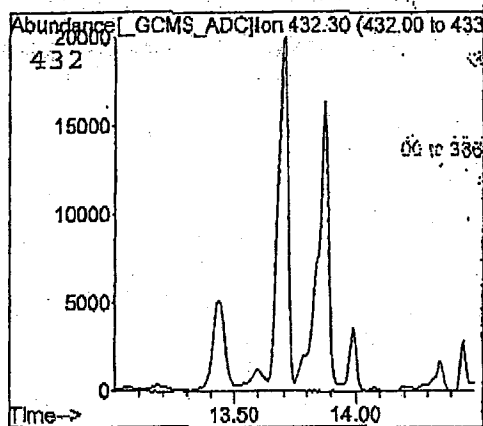
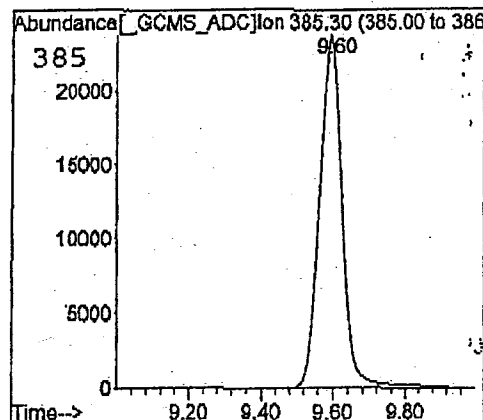
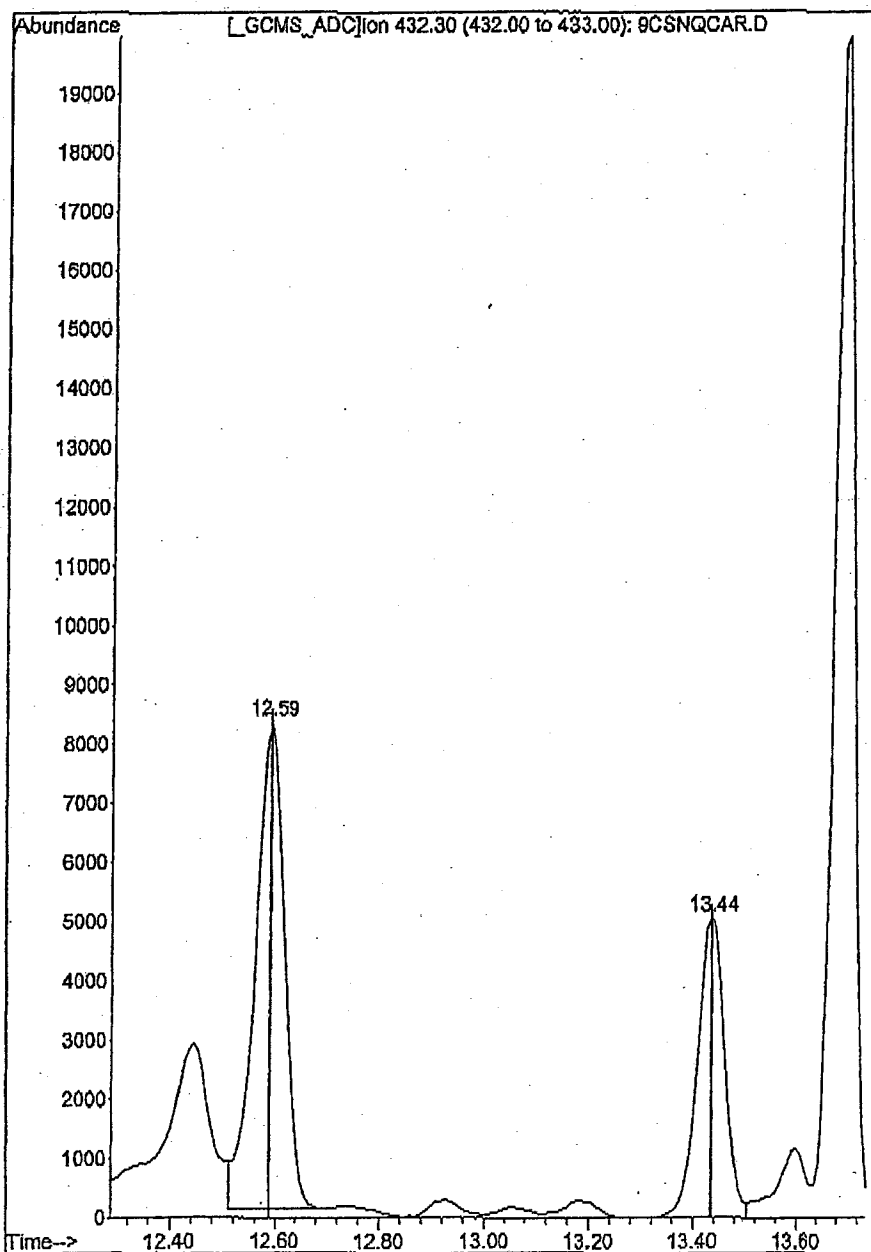
UCLA OLYMPIC ANALYTICAL LABORATORY

p 6

Sample Name : TE NEGATIVE QC
Data File : 9CSNQCAR.D
Miscellaneous:
Equipment # : MSDA13
Method File : TESIM04A
Analysis Time: 29 Jun 2006 1:43 pm
ALS Bottle # : 73

| | RT | RRT | PEAK HEIGHT |
|-------------------------|--------|------|-------------|
| Internal Standard (385) | 9.595 | 1.00 | 23767 |
| Testosterone (432) | 13.435 | 1.40 | 5097 |
| Epitestosterone (432) | 12.589 | 1.31 | 8407 |

Testosterone/Epitestosterone Peak Height Ratio = 0.606



11/30/06

1/30/06 GDC01362.6

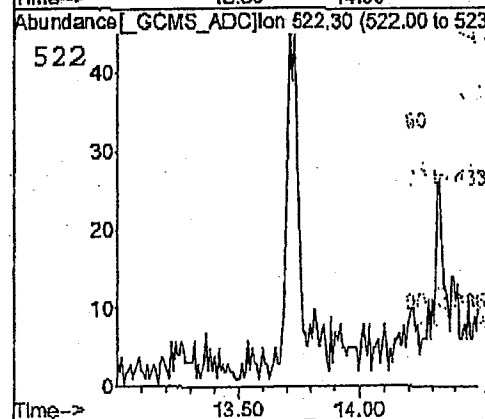
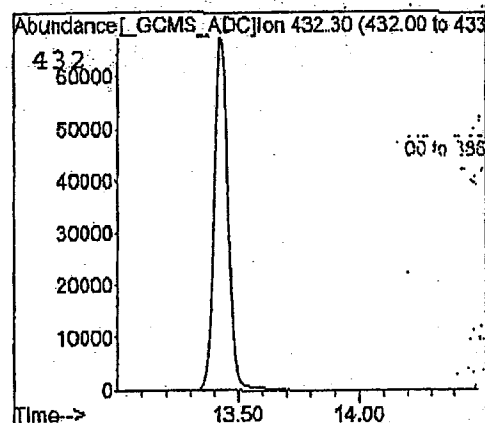
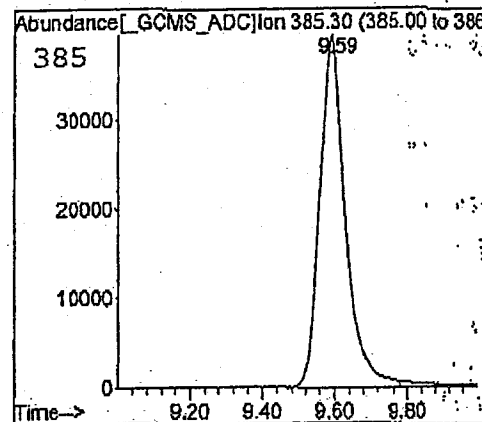
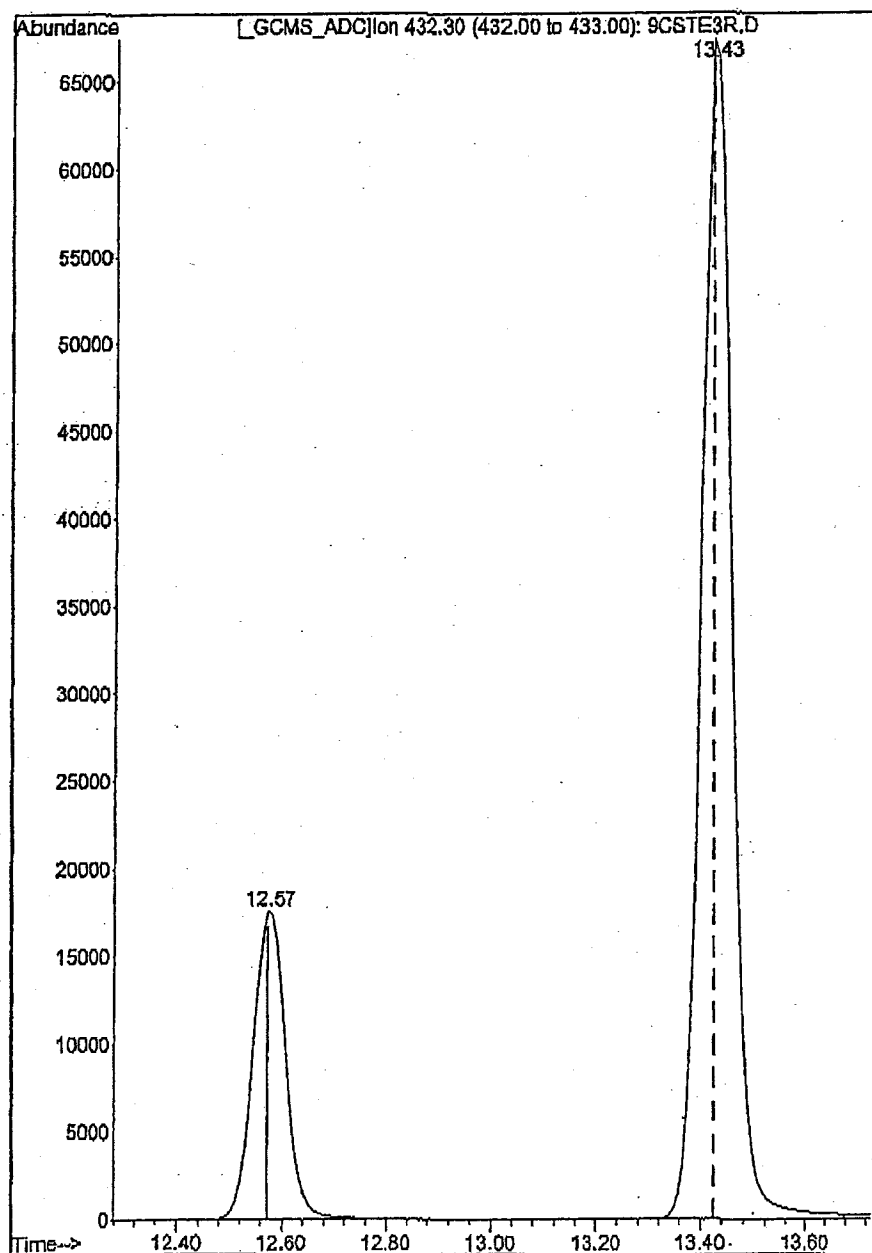
UCLA OLYMPIC ANALYTICAL LABORATORY

p 7

Sample Name : TE 4:1 STANDARD
 Data File : 9CSTE3R.D
 Miscellaneous:
 Equipment # : MSDA13
 Method File : TESIM04A
 Analysis Time: 29 Jun 2006 8:15 pm
 ALS Bottle # : 86

| | RT | RRT | PEAK HEIGHT |
|-------------------------|--------|------|-------------|
| Internal Standard (385) | 9.594 | 1.00 | 39563 |
| Testosterone (432) | 13.427 | 1.40 | 67499 |
| Epitestosterone (432) | 12.572 | 1.31 | 17643 |

Testosterone/Epitestosterone Peak Height Ratio = 3.826



Mel/30/02 6/30/02 DC01362.7

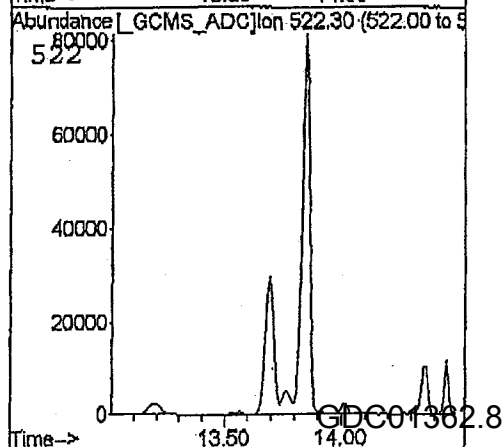
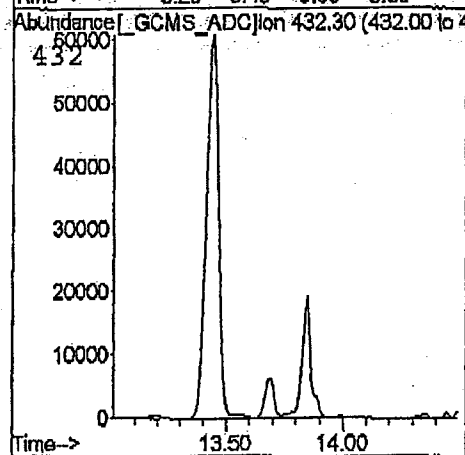
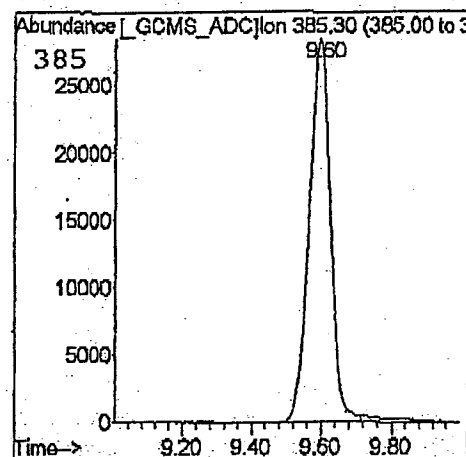
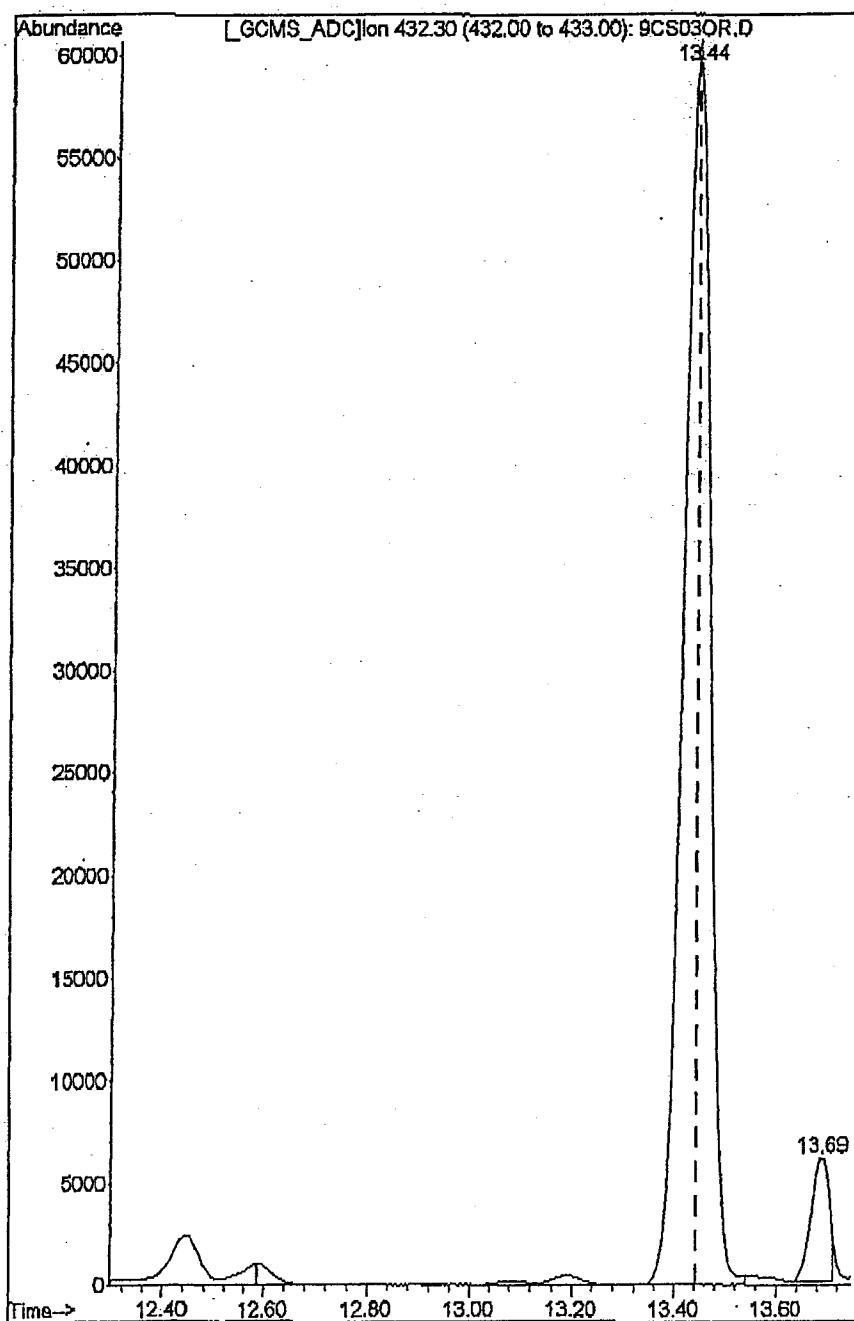
UCLA OLYMPIC ANALYTICAL LABORATORY

Sample Name : 9C E A CONFIRMATION
 Data File : 9Cb
 Miscellaneous:
 Equipment # : MSDA13
 Method File :
 Analysis Time: 4:21 pm
 ALS Bottle # : 79

p 8

| | RT | RRT | PEAK HEIGHT |
|-------------------------|--------|------|-------------|
| Internal Standard (385) | 9.594 | 1.00 | 28498 |
| Testosterone (432) | 13.444 | 1.40 | 60727 |
| Epitestosterone (432) | 12.589 | 1.31 | 1081 |

Testosterone/Epitestosterone Peak Height Ratio = 56.177



12/11

| DATE | SAMPLE | T | E | T/E (peak area) | Andro- 11keto | Etio- 11keto | 5aDiol- 5pDiol | 5bDiol- 5pDiol |
|---------|---------|------|------|-----------------------|------------------|-----------------|-------------------|-------------------|
| 7/3/06 | 995462 | 9.6 | 3.2 | 2.8 | -0.22‰ | -0.95‰ | n/r | -1.04‰ |
| 7/11/06 | 994203 | 15 | 13.6 | 1.3 | -0.25‰ | -1.29‰ | -2.91‰ | -1.05‰ |
| 7/13/06 | 994277 | 16.6 | 7.6 | 2.5 | -2.32‰ | -1.99‰ | -4.62‰ | --4.09‰ |
| 7/14/06 | 994276 | 16.2 | 13.1 | 1.5 | -1.70‰ | -1.04‰ | -1.01‰ | -0.70‰ |
| 7/18/06 | 994075 | 20 | 11.8 | 1.8 | -1.22‰ | -1.89‰ | -5.06‰ | -3.56‰ |
| 7/20/06 | 995474B | 47.5 | 4.4 | 11 | -3.51‰ | -2.02‰ | -6.39‰ | -2.65‰ |
| 7/22/06 | 994080 | 20 | 9.6 | 2.5 | -1.36‰ | -1.68‰ | -4.80‰ | -1.67‰ |
| 7/23/06 | 994171 | 8.5 | 9.2 | 1 | -0.64‰ | -1.43‰ | -4.96‰ | -1.45‰ |

Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (12)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

Sport und Buch Strauß, Köln, 2004

H. GEYER, U. FLENER, U. MARECK, F. SOMMER, W. SCHÄNZER:
Preliminary Results regarding the detection of the misuse of testosterone gel
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping
analysis (12). Sport und Buch Strauß, Köln (2004) 121-127

Preliminary results regarding the detection of the misuse of testosterone gel

Institute of Biochemistry, German Sport University Cologne, Germany

*Clinic and Polyclinic of Urology, University Hospital Cologne, Germany

Abstract

Since 2003 testosterone gel is admitted as therapeutic to the German market. Testosterone gel is used for the treatment of testosterone deficiency in males. To analyse the influence of a testosterone gel application on the urinary steroid profile and the carbon isotope ratios of testosterone metabolites, urine samples were collected from 2 patients, who were treated with testosterone gel for several weeks (100 mg testosterone per day). The urine samples were analysed for endogenous steroids with GC/MS and GC/C/IRMS.

The following main results were obtained: The testosterone/epitestosterone (T/E) ratios increased mainly by a decrease of the epitestosterone concentrations. After 8 days of treatment the maximum T/E ratios were 7,7 (patient 1) and 13,4 (patient 2). During the treatment the T/E ratios showed stable values with normal variations during the day. The steroid profiles of the 2 patients looked like profiles of individuals with natural increased T/E ratios based on a decreased epitestosterone excretion. The GC/C/IRMS analyses of the testosterone metabolites androsterone and etiocholanolone gave suspicious but not fully conclusive results. The results show that it is difficult or even impossible to prove the misuse of testosterone gel with the actually used methods (steroidprofiling, longitudinal studies, GC/C/IRMS).

Introduction

According to the WADA-list of prohibited substances, the use of testosterone is prohibited in sports (1). Since the year 2000 a new testosterone preparation, testosterone gel, is available on the US market and since 2003 on the European market. Testosterone gel is used for replacement therapy in men for conditions associated with low testosterone. The gel is administered by application on the skin. The daily dose is 25-100 mg testosterone and about 9-14 percent of the testosterone is absorbed through the skin (2). It has been shown that the application of testosterone gel leads to constantly increased serum-testosterone values (3). Additionally sev-

GDC01364.2

eral investigations have shown, that the application of testosterone gel leads to performance enhancing physiological effects like significant increase of the lean body mass, muscle strength and hemoglobin concentration (4). The objective of the following investigation was to find out, if an application of testosterone gel can be detected with the actual methods used in doping control.

Experimental

Two 43 years old male patients suffering from testosterone deficiency were treated 21 days with testosterone gel (Testogel®, Jenapharm, Germany, daily application of 2 sachets with 5g testosterone gel each; total testosterone amount per day = 100mg). Before and during the treatment serum testosterone and serum LH were analysed according to standard operating procedures of the Clinic and Polyclinic of Urology of the University Hospital Cologne. Additionally urine samples were collected for 24 hours before the administration of testosterone gel and 8 days after daily application of testosterone gel. The urine samples were analysed with gaschromatography/mass spectrometry for the profiles of endogenous steroids according to a modified method from Donike et al. (5, 6). Additionally the urine samples were analysed with carbon isotope mass spectrometry (GC/C/IRMS) to determine the $^{12}\text{C}/^{13}\text{C}$ carbon isotope ratios of the testosterone metabolites androsterone and etiocholanolone. The GC/C/IRMS analyses were performed according to a method from Flenker et al. (7, 8).

Results and discussion

After a daily treatment with 100 mg testosterone via a transdermal application of testosterone gel the serum testosterone concentrations of the two patients increased within 6 days from 3 to 9 ng/ml and 2 to 6 ng/ml respectively. The serum LH values decreased for patient 1 from 5.5 U/l to 1.0 U/l.

After 8 days of treatment with testosterone gel the urinary testosterone/epitestosterone (T/E)-ratios of the 2 patients increased from values of 1.3 ± 0.15 and 1.0 ± 0.09 to values of 5.3 ± 1.6 and 8.9 ± 3.8 (see fig. 1).

The increase of the T/E ratio was mainly based on a decrease of the epitestosterone concentrations and not on an increase of the testosterone concentrations (see fig. 2). The testosterone concentrations did not exceed the upper limits of the reference ranges of athletes (9). During the treatment the T/E ratios showed stable values with normal variations during the day and within several days (see fig. 1) although testosterone gel was administered every morning.

This is totally different to the oral or intramuscular application of testosterone which is followed by a strong increase of the T/E ratios and strong variations of the T/E ratios during the day (fig. 3 and 4). Based on our longtime experience in the evaluation of endocrinological and longitudinal studies the steroid profiles of the 2 patients looked like profiles of individuals with natural increased T/E ratios based on a decreased epitestosterone excretion. Based on the steroid profile data we would not give such a sample positive. An athlete, who would administer testosterone gel could keep his T/E ratios on a constant level for a long time period, so that the misuse cannot be detected by a longitudinal study or endocrinological study.

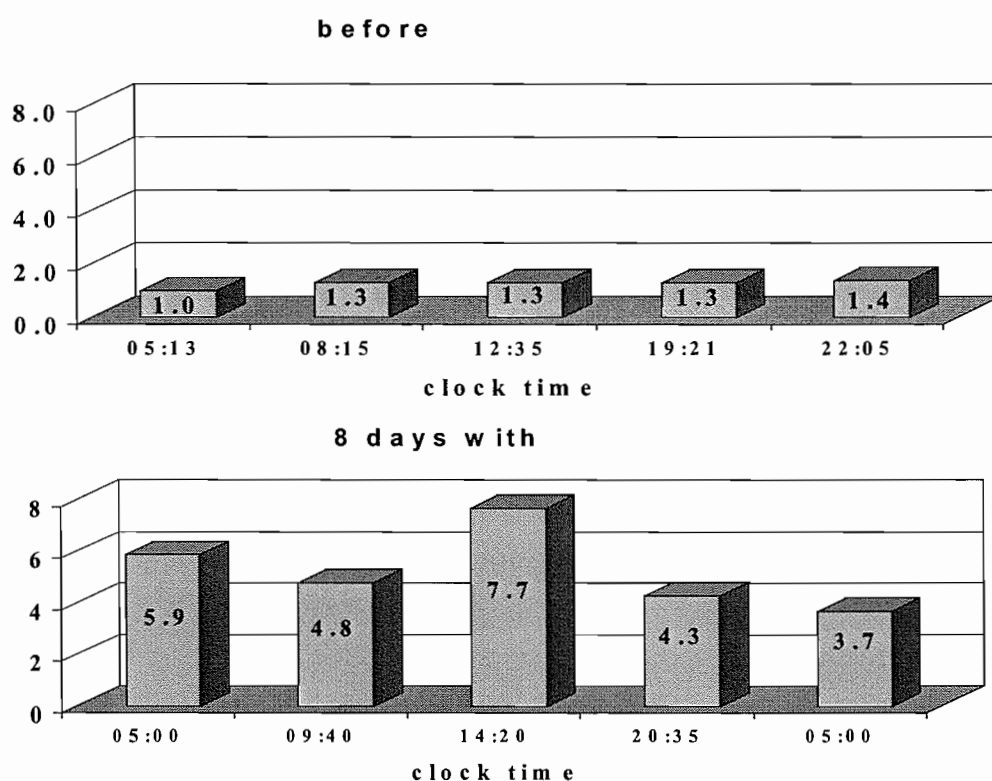


Fig.1 : T/E ratios before and 8 days after the daily treatment with testosterone gel (patient 1; 100 mg testosterone per day; application at 8:00)

The GC/C/IRMS analyses of the testosterone metabolites androsterone and etiocholanolone in urine samples of patient 1 before and 8 days after the daily application of testosterone gave the following results:. The $\delta^{13}\text{C}$ values of etiocholanolone decreased from -24.8 ‰ to -26.3 ‰ and the $\delta^{13}\text{C}$ values of androsterone decreased from -23.1 ‰ to -25.4 ‰ . According to our actual criteria only a decrease of at least two $\delta^{13}\text{C}$ units for an-

drosterone and three $\delta^{13}\text{C}$ units for etiocholanolone is consistent with an application of testosterone or testosterone prohormones. Therefore the GC/CIRMS analyses delivered only suspicious but not fully conclusive results for an application of testosterone.

One reason for this weak shift of the ^{13}C values of the testosterone metabolites is that the testosterone in the gel has only a $\delta^{13}\text{C}$ value of -27‰ and therefore is not as depleted as prohormones with the $\delta^{13}\text{C}$ values of about -30‰ .

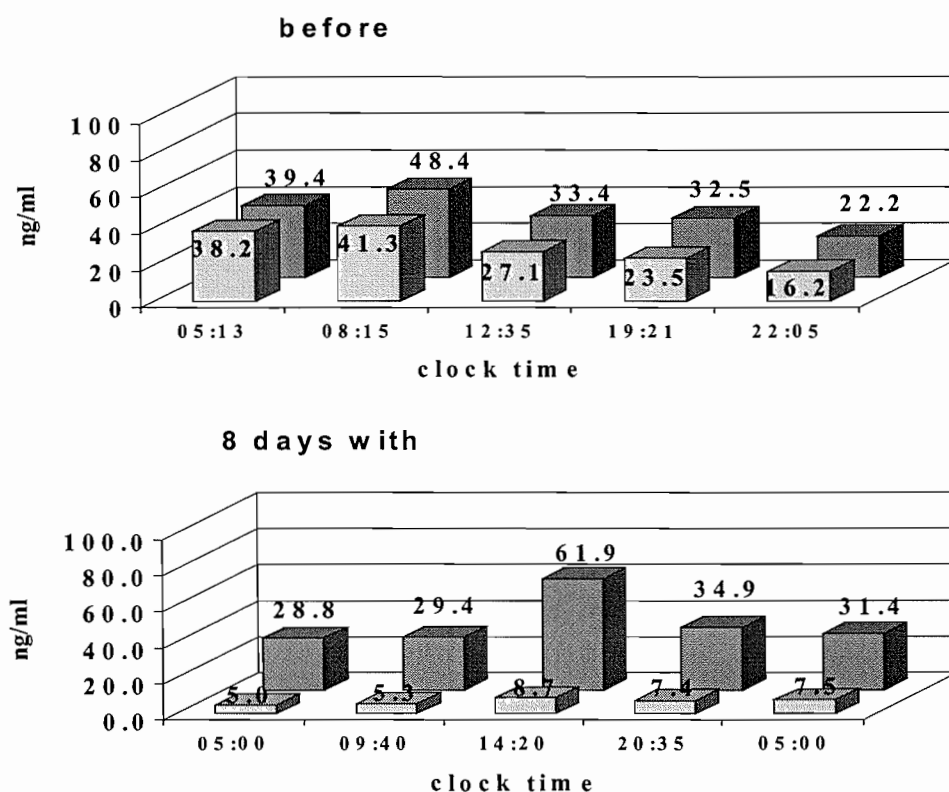


Fig. 2: Urinary concentrations of testosterone (rear bars) and epitestosterone (front bars) before and 8 days after the daily treatment with testosterone gel (patient 1; 100 mg testosterone per day; application at 8:00)

Conclusion

The results show that it is difficult or even impossible to prove the misuse of testosterone gel with the actually used methods (steroidprofiling, longitudinal studies, GC/C/IRMS). To prevent the misuse of this new preparation by athletes it is necessary to develop new methods or combine several known methods for its detection.

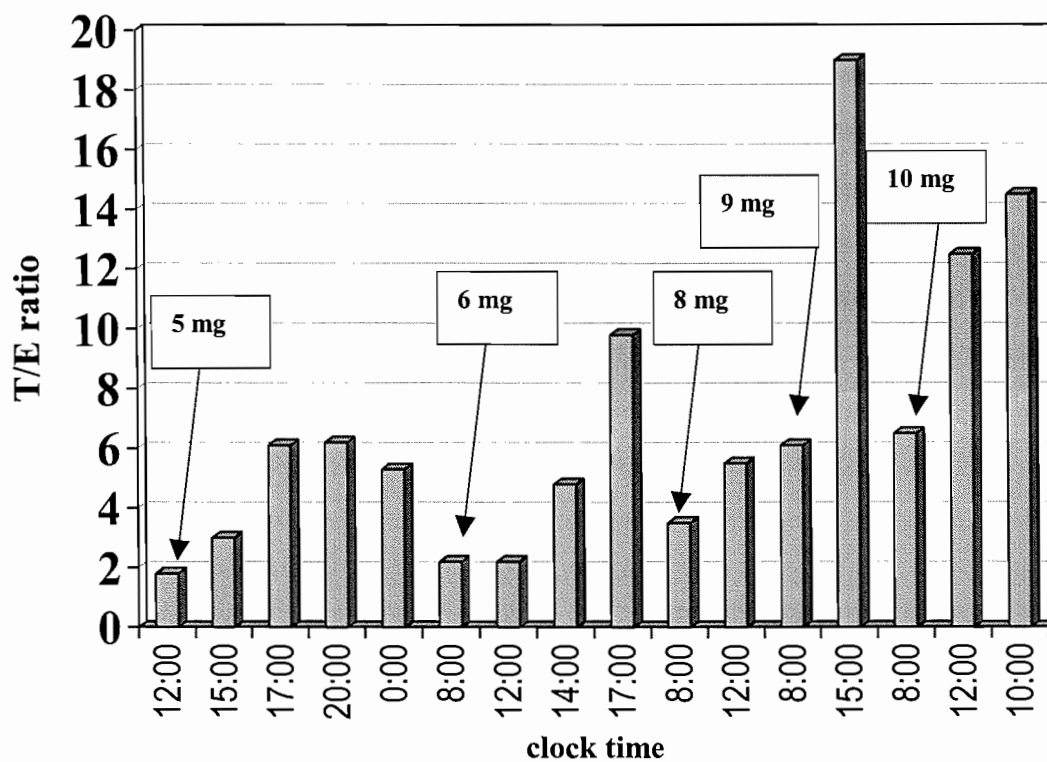


Fig. 3: T/E ratios after repeated injection of different amounts of testosterone propionate (13).

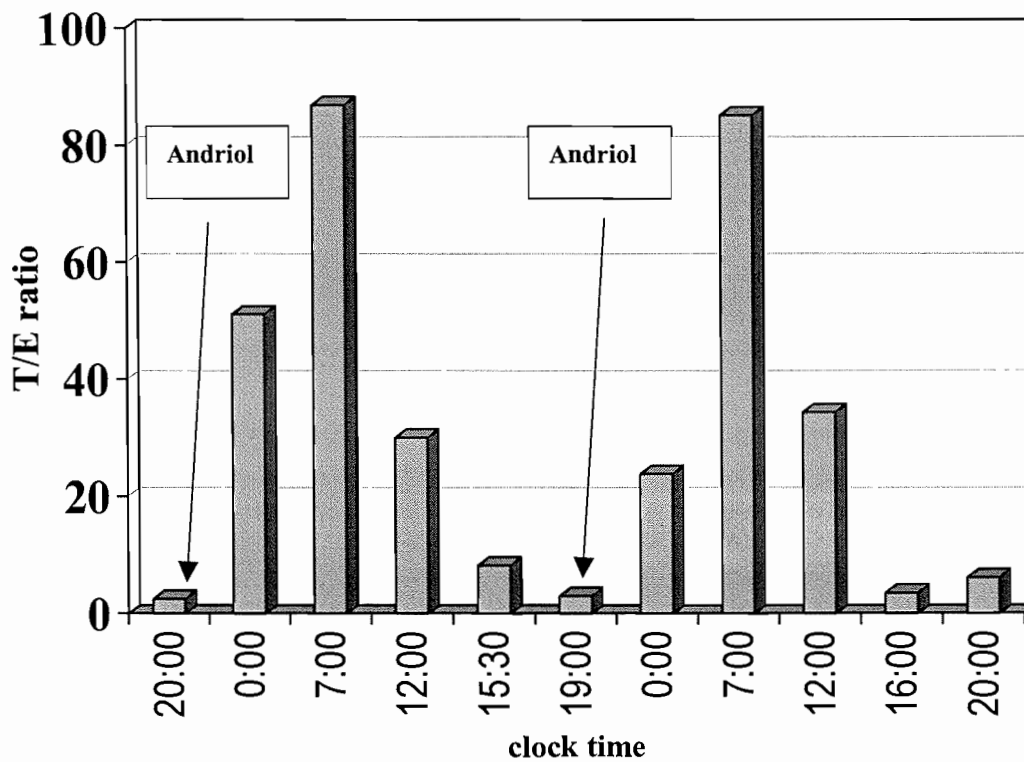


Fig. 4: T/E ratios after repeated oral application of 40 mg testosterone undecanoate (Andriol®) (14)

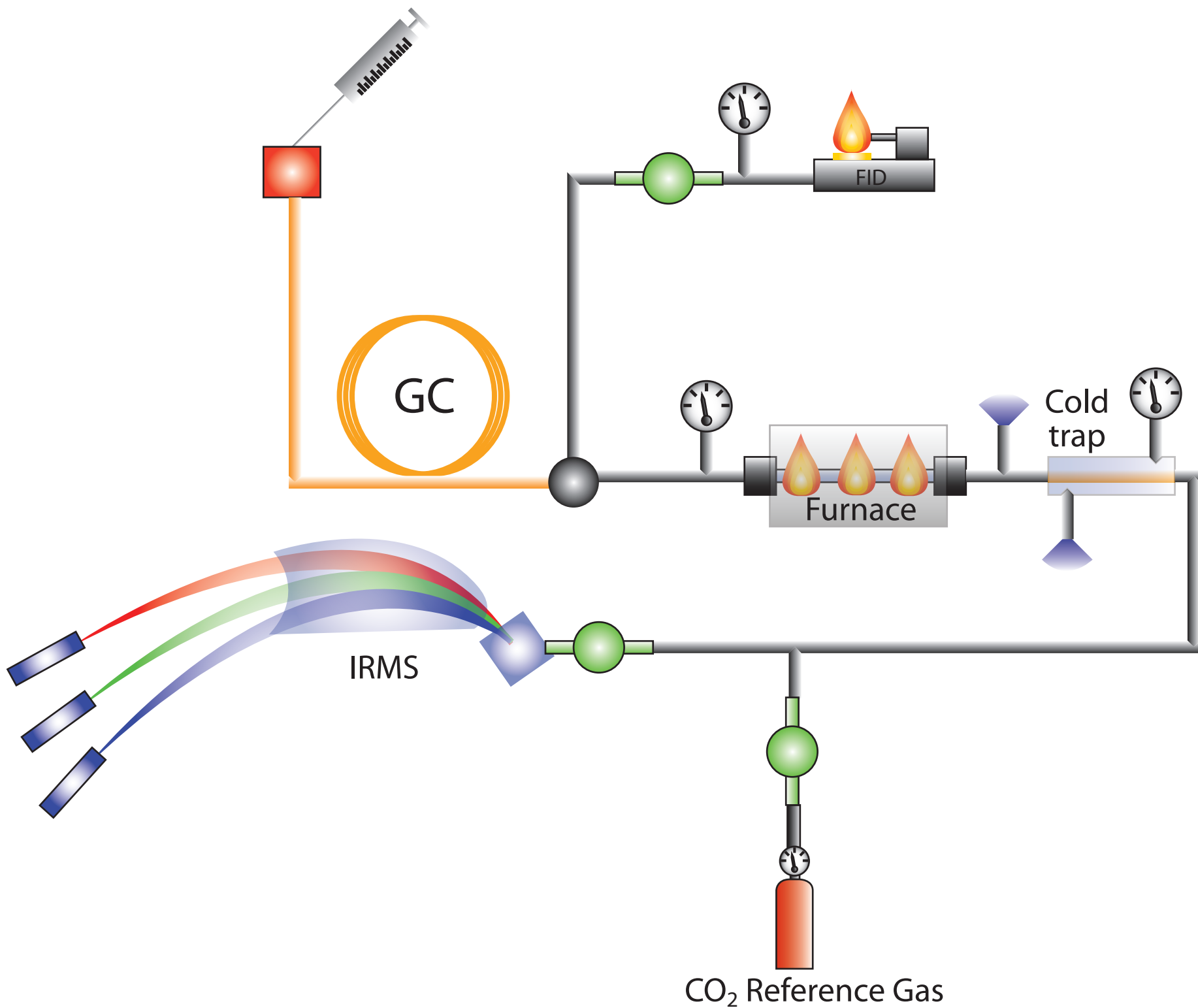
References

1. World Anti Doping Agency. The 2004 prohibited list. International Standard. WADA, Montreal (2004)
2. Jenapharm: Produktmonographie Testogel. Jenapharm, Jena (2003)
3. Jockenhövel, F.: Männlicher Hypogonadismus-Aktuelle Aspekte der Hormonsubstitution. Uni-Med Verlag, Bremen (2003)
4. Swerdloff, R.S., Wang, C., Cunningham, G., Dobs, A., Iranmanesh, A., Matsumoto, A.M., Snyder, P., Weber, T., Longstreth, J., Berman, N.: Longterm pharmacokinetics of transdermal testosterone gel in hypogonadal men. *J Clin Endocrinol Metab* 85 (2000) 4500-10
5. Donike, M., Ueki, M., Kuroda, Y., Geyer, H., Nolteernsing, E., Rauth, S., Schänzer, W., Schindler, U., Völker, E., Fujisaki, M.: Detection of dihydro-testosterone (DHT) doping - Alterations in the steroid profile and reference ranges for DHT and its 5 α -metabolites. *J. Sports.Med.Phys.Fitness*, 35 (1995) 235-50.
6. Geyer, H., Schänzer, W., Mareck-Engelke, U., Nolteernsting, E., Opfermann, G.: Screening procedure for anabolic steroids-The control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) *Recent advances in doping analysis* (5). Sport und Buch Strauß, Köln (1998) 99-102
7. Flenker, U., Nolteernsting, E., Geyer, H., Schänzer, W.: A Screening method for synthetic testosterone and its precursors based on GC/C/IRMS. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) *Recent advances in doping analysis* (7). Sport und Buch Strauß, Köln, (1999) 241-248
8. Flenker, U., Horning, S., Nolteernsting, E., Geyer, H., Schänzer, W.: Measurement of $^{12}\text{C}/^{13}\text{C}$ -Ratios to Confirm Misuse of Endogenous Steroids. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) *Recent advances in doping analysis* (6). Sport und Buch Strauß, Köln (1999) 243-256
9. Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M.: The Cologne protocol to follow up high testosterone/ epitestosterone ratios. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) *Recent advances in doping analysis* (4). Sport und Buch Strauß, Köln, (1997) 107-126

10. Geyer, H., Flenker, U., Mareck, U., Sommer, F., Schänzer, W.: Preliminary results regarding the detection of the misuse of testosterone gel. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (12). Sport und Buch Strauß, Köln (2004) submitted for publication
11. Güntner, U., Flenker, U., Ayotte, C., Ueki, M., Schänzer, W.: D/H Isotope Analysis of Endogenous Steroids -First Results-. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (12). Sport und Buch Strauß, Köln (2004) submitted for publication
12. Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M.: The Cologne protocol to follow up high testosterone/ epitestosterone ratios. In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (4). Sport und Buch Strauß, Köln , (1997) 107-126
13. Geyer, H., Donike, M.: Testosterone/epitestosterone ratios after repeated injection of different amounts of testosterone propionate. Unpublished results (1992)
14. Schänzer, W., Geyer, H., Mareck, U.: Testosterone/epitestosterone ratios after repeated oral application of testosterone undecanoate. Unpublished results (1996)

Changes During Reprocessing

| | | New Point Added To Background Trace | Exisiting Background Point Dragged To New Point | Number Of Times Sample Was Re-processed | Number Of Times Peak Start / End Was Changed |
|---|-------------------------------------|-------------------------------------|---|---|--|
| A | Reprocessing by Mongongou | | | | |
| | 178/07 995474 F3/45 ul in 2 ul | 3 | 1 | 3 | 2 |
| | Blu 1 pool 4 F1 / 50 ul inj 1 ul | 2 | 3 | 1 | 1 |
| | 178/07 995474 1/150 ul inj 1ul | 7 | 5 | 5 | 2 |
| | Blu 1 Pool 4 F2 500 ul inj 1ul | 3 | 1 | 3 | 1 |
| | 178/07995474 F2 / 400 ul 1 inj 1 ul | 2 | 1 | 2 | 2 |
| | MixCal Acetate 001 A-2 230706 | 5 | | 1 | 2 |
| B | Reprocessing by Frelat | | | | |
| | MixCal Acetate 001A | 5 | 4 | 4 | 2 |
| 1 st attempt | Blu 1 Pool 4 F3 / 45 ul inj 2 ul | 5 | | 6 | |
| Could not make numbers change. Closed manual DP, re-loaded sample, re-started reprocessing | | | | | |
| 2 nd attempt | Blu 1 Pool 4 F3 / 45 ul inj 2 ul | 10 | 9 | 4 | |
| Still could not make numbers change | | | | | |
| 3 rd attempt | Blu 1 Pool 4 F3 / 45 ul inj 2 ul | 4 | 8 | 2 | 4 |
| 1 st attempt | 178/07 995474 F3/45 ul inj 2ul | 7 | 9 | 2 | 1 |
| Processing re-started by Frelat | | | | | |
| 2 nd attempt | 178/07 995474 F3/45 ul inj 2ul | 3 | 4 | 3 | 2 |
| | Blu 1 pool 4 F1 120ul inj 2 ul | 3 | 2 | 2 | 1 |
| | 178/07 995474 F1 / 150 ul inj 2 ul | 3 | 4 | 4 | 1 |
| | Blu 1 pool 4 F2 / 1 400 ul inj 2 ul | 2 | 2 | 3 | 2 |
| | 17807 995474 F2 / 850 ul inj 2 ul | 4 | 4 | 1 | 0 |
| | Mix Cal Acetate 001A-100ng inj | 2 | 3 | 1 | 3 |



| | | | | |
|-----------------|------------|-------------|-------|----|
| Largest | 7.1686E-03 | sd (per ml) | 0.070 | |
| Smallest | 7.1681E-03 | | | |
| Largest | 1.1988E-04 | sd (per ml) | 1.755 | |
| Smallest | 1.1967E-04 | | | |
| Largest | 1.1969E-04 | sd (per ml) | 2.429 | |
| Smallest | 1.1940E-04 | | | |
| Largest | 1.2353E-04 | sd (per ml) | 3.330 | 1 |
| Smallest | 1.2312E-04 | | | |
| Largest | 1.2453E-04 | sd (per ml) | 4.112 | 2 |
| Smallest | 1.2402E-04 | | | |
| Largest | 1.2471E-04 | sd (per ml) | 4.753 | 3 |
| Smallest | 1.2412E-04 | | | |
| Largest | 1.2491E-04 | sd (per ml) | 2.649 | 4 |
| Smallest | 1.2458E-04 | | | |
| Largest | 1.2514E-04 | sd (per ml) | 1.521 | 5 |
| Smallest | 1.2495E-04 | | | |
| Largest | 1.2512E-04 | sd (per ml) | 1.361 | 6 |
| Smallest | 1.2495E-04 | | | |
| Largest | 1.2517E-04 | sd (per ml) | 1.841 | 7 |
| Smallest | 1.2494E-04 | | | |
| Largest | 1.2510E-04 | sd (per ml) | 2.002 | 8 |
| Smallest | 1.2485E-04 | | | |
| Largest | 1.2525E-04 | sd (per ml) | 1.519 | 9 |
| Smallest | 1.2506E-04 | | | |
| Largest | 1.2532E-04 | sd (per ml) | 1.198 | 10 |
| Smallest | 1.2517E-04 | | | |
| Largest | 1.2523E-04 | sd (per ml) | 1.039 | 11 |
| Smallest | 1.2510E-04 | | | |
| Largest | 1.2529E-04 | sd (per ml) | 1.439 | 12 |
| Smallest | 1.2511E-04 | | | |
| Largest | 1.2482E-04 | sd (per ml) | 1.766 | 13 |
| Smallest | 1.2460E-04 | | | |

| Ratio Results | Ratio | sd (per ml) | Peak height (nA) | Range of test (nA) | nA per mil |
|-------------------------|------------|-------------|------------------|--------------------|------------|
| Linearity 1 26/06/06 | 1.1792E-02 | 0.339 | 9.3 | 7.8 | 0.044 |
| | 1.1788E-02 | | 1.5 | | |
| Linearity 2 26/06/06 | 1.1792E-02 | 0.339 | 9.8 | 8.3 | 0.041 |
| | 1.1788E-02 | | 1.5 | | |
| Linearity 3 26/06/06 | 1.1790E-02 | 0.170 | 9.9 | 8.4 | 0.020 |
| | 1.1788E-02 | | 1.5 | | |
| Linearity 1 31/07/06 | 1.7790E-02 | 1.689 | 9.8 | 8.2 | 0.206 |
| | 1.7760E-02 | | 1.6 | | |
| Linearity 2 31/07/06 | 1.1778E-02 | 0.170 | 10 | 8.4 | 0.020 |
| | 1.1776E-02 | | 1.6 | | |
| Linearity 3 31/07/06 | 1.1777E-02 | 0.170 | 10 | 8.4 | 0.020 |
| | 1.1775E-02 | | 1.6 | | |

Isoprime Linearity Specifications

Source: GVI website page
<http://www.gvinstruments.co.uk/isoprime7.htm>

Continuous Flow Preparation Systems

Analyser

Performed using a reference gas box and continuous flow interface. Applies to all continuous flow modules.

Reference gas precision - ^{13}C and ^{15}N

0.1 ‰ (SD 1 : on the fit of 10 consecutive pulses of 5 nA height)

Reference gas linearity - ^{13}C and ^{15}N

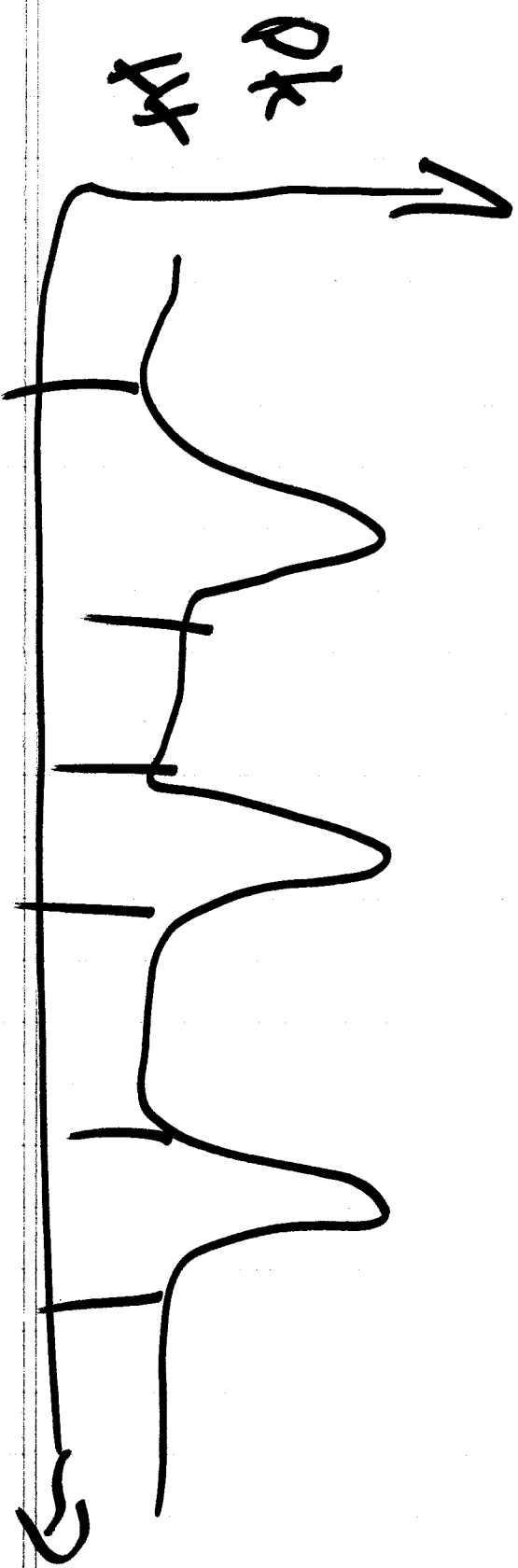
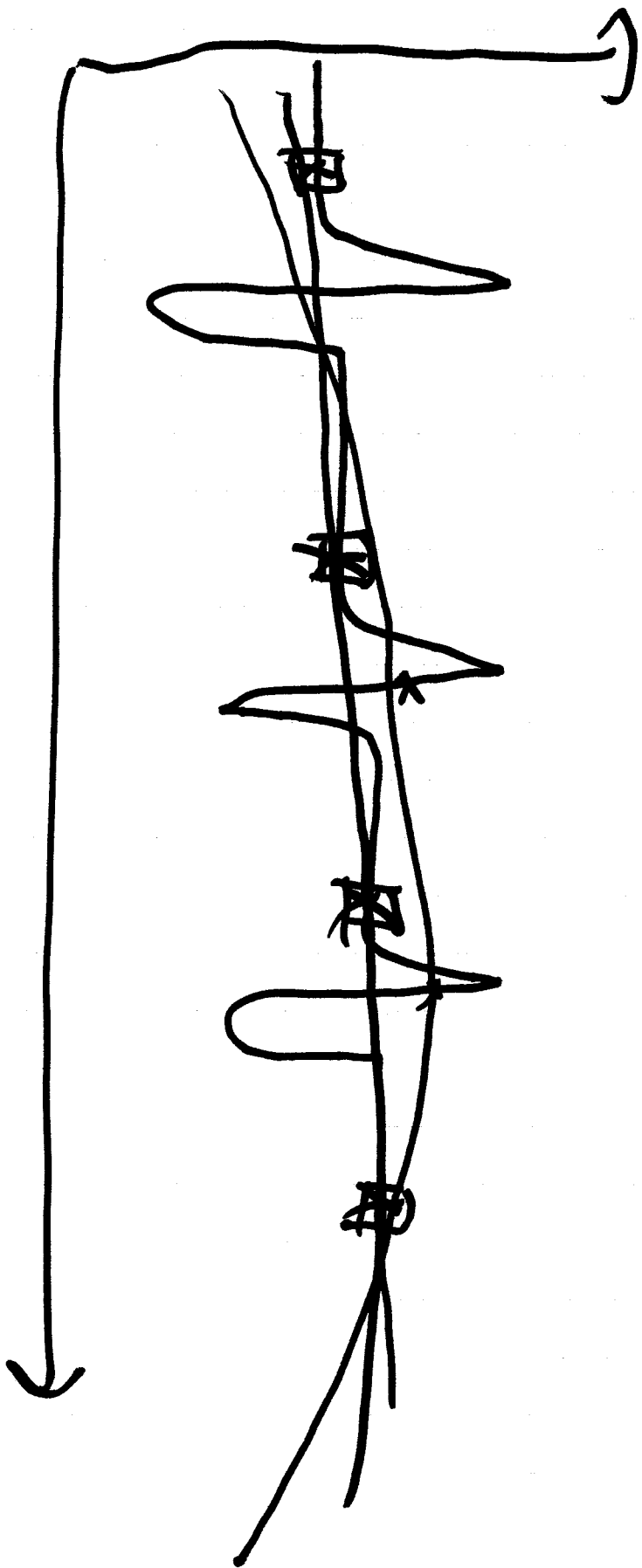
0.3 ‰ (SD 1 : 10 pulses varying in height between 1-10 nA)

Thermo Linearity Specifications

| Sensitivity | 1100 M/I | 1500 M/I |
|--|---------------|---------------------|
| Continuous Flow mode | | |
| Molecules CO_2 / mass 44 ion with | | |
| Isotope Ratio Linearity* | | 0.02 ‰ / nA |
| Mass Range at 3 kV | 1 – 96 Dalton | 1 – 80 Dalton |
| Mass Resolution $m/\Delta m$ (10 % valley) | | 110 |
| Effective Magnetic Deflection Radius | | 191 mm |
| System Stability | | < 10 ppm |
| H_3^+ Factor | | < 10 ppm / nA |
| H_3^+ Factor Stability | | < 0.03 ppm / nA / h |
| Noise Level | | < 50 dB(A) |

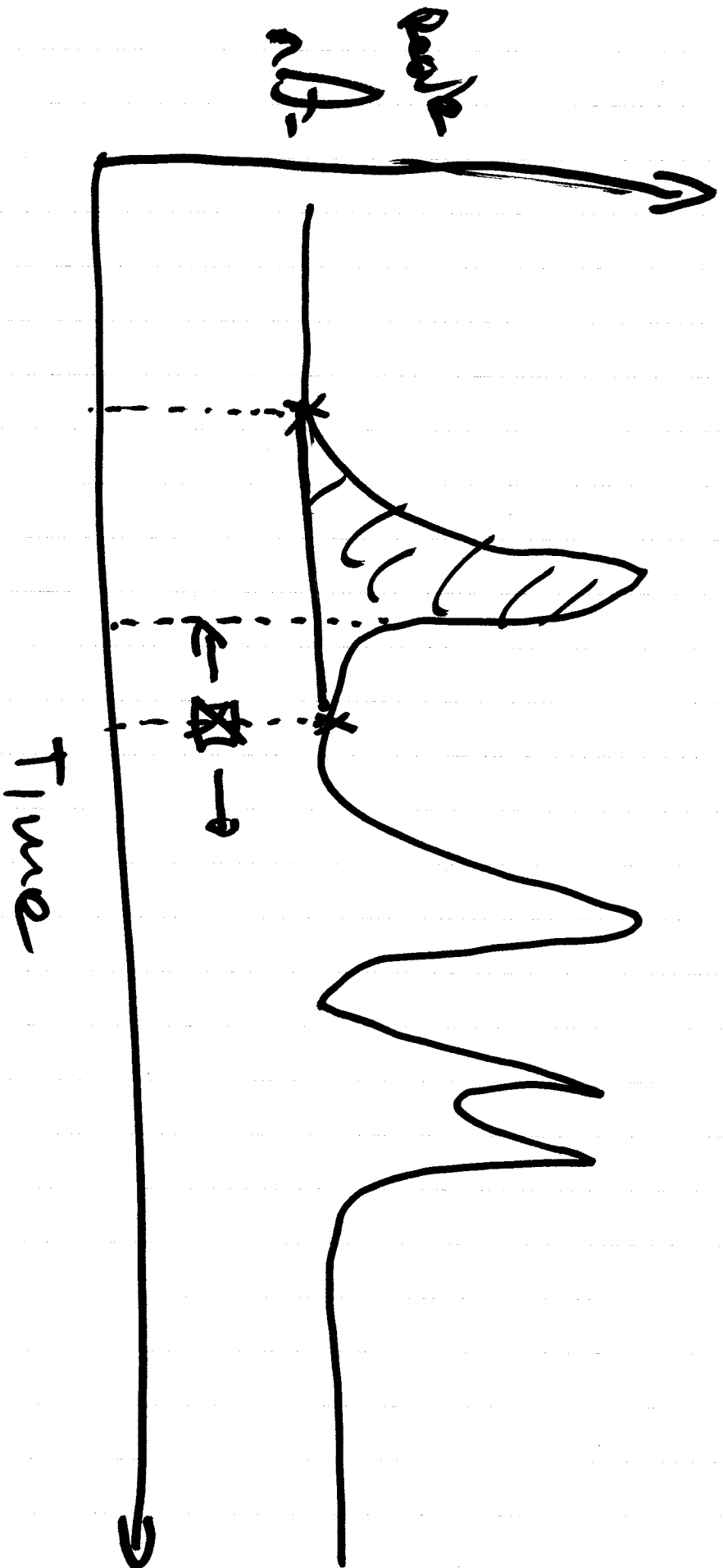
*Known as Ion Source Linearity

Source: thermo product sheet
 "productPDF_26513"



Time

View
Dros



HAEMATOCRIT TEST RESULTS FOR RIDER

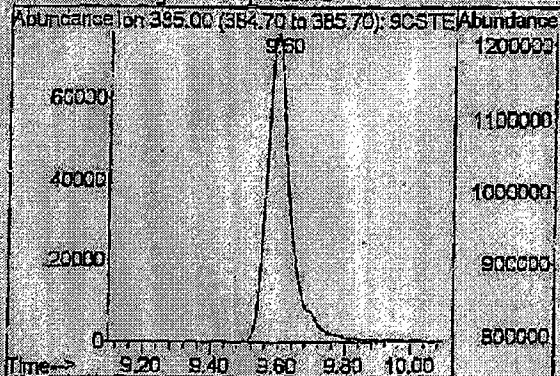
NAME: LANDIS Floyd
 UCI CODE: USA1975101
 SORTED BY: DATE
 NUMBER OF RECORDS: 8
 PERIOD COVERED: 01.01.2005 TO 08.08.2006

| RIDER | | RACE | | | | INSPECTOR | | | | | | | |
|-------|--------------|------|-------------|-----------------------|---------------------------|-----------|------------|---------------------|---------------------|------|------|------|-------|
| NUM | NAME | SEX | UCI CODE | TEAM NAME | NAME | DISC | DATE | PLACE | NAME | HCT | HB | RETI | DECIS |
| 35520 | LANDIS Floyd | Male | USA19751014 | PHONAK HEARING SYSTEM | PARIS-NICE | ROA | 06.03.2005 | Paris | VANDEVYVERE Marc | 46.8 | 15.6 | 0.98 | APTE |
| 36810 | LANDIS Floyd | Male | USA19751014 | PHONAK HEARING SYSTEM | TOUR DE France - GENERAL | ROA | 30.06.2005 | Nantes - France | BRUN Martin | 44.5 | 14.8 | 1.07 | APTE |
| 37498 | LANDIS Floyd | Male | USA19751014 | PHONAK HEARING SYSTEM | TOUR DE France | ROA | 18.07.2005 | Pau - France | VANDEVYVERE Marc | 45.2 | 15.3 | 0.45 | APTE |
| 37529 | LANDIS Floyd | Male | USA19751014 | PHONAK HEARING SYSTEM | TOUR DE France | ROA | 23.07.2005 | St-Etienne - France | VANDEVYVERE Marc | 42.8 | 14.5 | 0.74 | APTE |
| 38976 | LANDIS Floyd | Male | USA19751014 | PHONAK HEARING SYSTEM | VIETA A ESPANA - controle | ROA | 25.08.2005 | Grenada - Espagne | PINTUELES Marie | 41.4 | 13.9 | 0.47 | APTE |
| 38782 | LANDIS Floyd | Male | USA19751014 | PHONAK HEARING SYSTEM | PARIS-NICE | ROA | 05.03.2006 | Paris - France | VANDEVYVERE Marc | 46.7 | 15.8 | 0.99 | APTE |
| 40791 | LANDIS Floyd | Male | USA19751014 | PHONAK HEARING SYSTEM | TOUR DE France - CONTROL | ROA | 29.06.2006 | Strasbourg - France | VOETS Jean-Michel | 44.8 | 15.5 | 1.3 | APTE |
| 41259 | LANDIS Floyd | Male | USA19751014 | PHONAK HEARING SYSTEM | TOUR DE France | ROA | 11.07.2006 | Mégnac - France | MERAVIGLIA Giovanni | 48.2 | 16.1 | 0.92 | APTE |

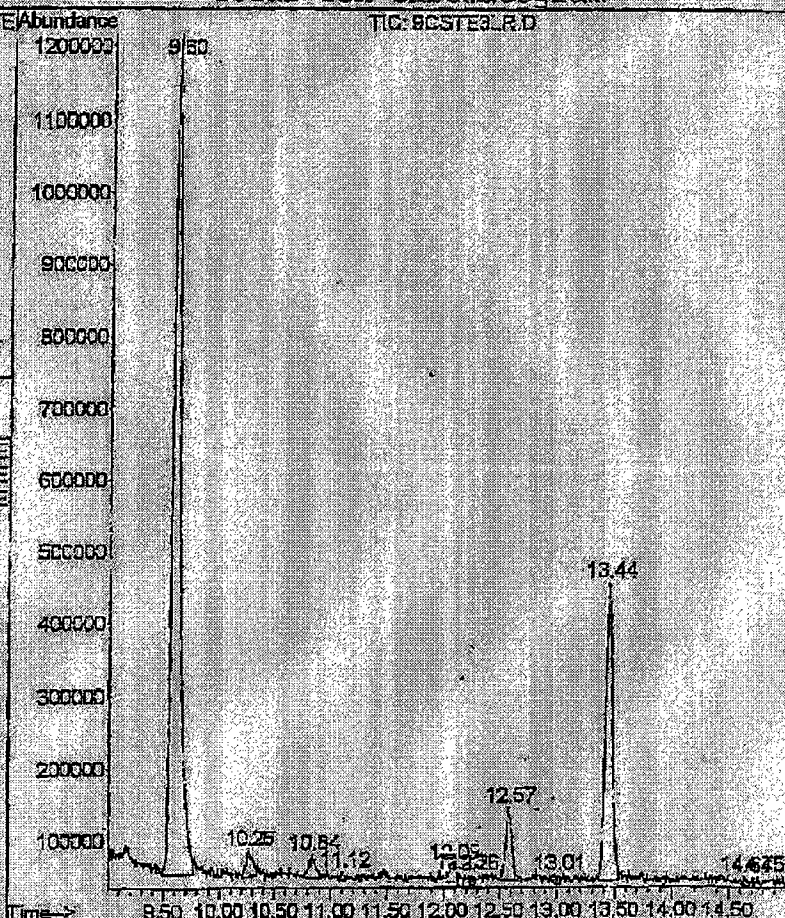
Data File : K:\CHEM\AAS\CONFIRM\MSDA13\9CSTE3LR.D
Acquired : 29 Jun 8:33 pm
Instrument : MSDA13
Misc Info :
Sample Name: TE 4:1 STANDARD LIN

Method File: ANAB97LS
Vial Number: 86

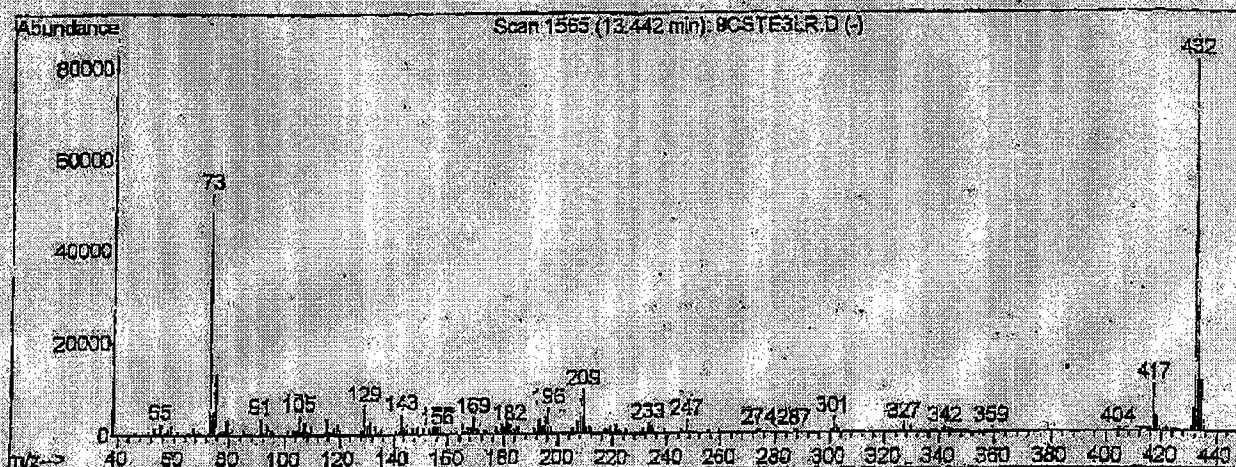
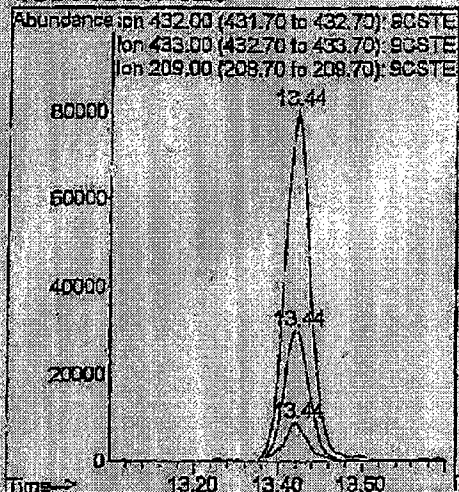
ISTD Ethylmorphine



Total Ion Chromatogram



Testosterone



Mulzol 6/30/00

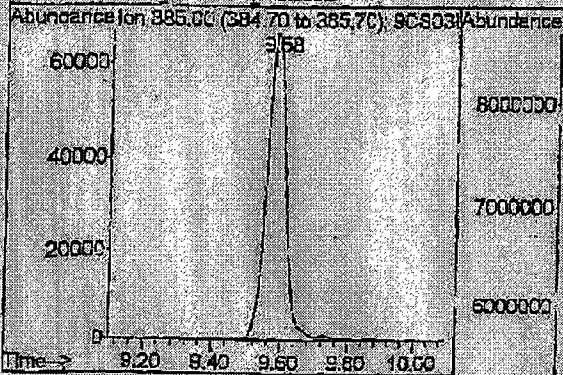
UCLA OLYMPIC ANALYTICAL LABORATORY
Testosterone Confirmation Report

p37

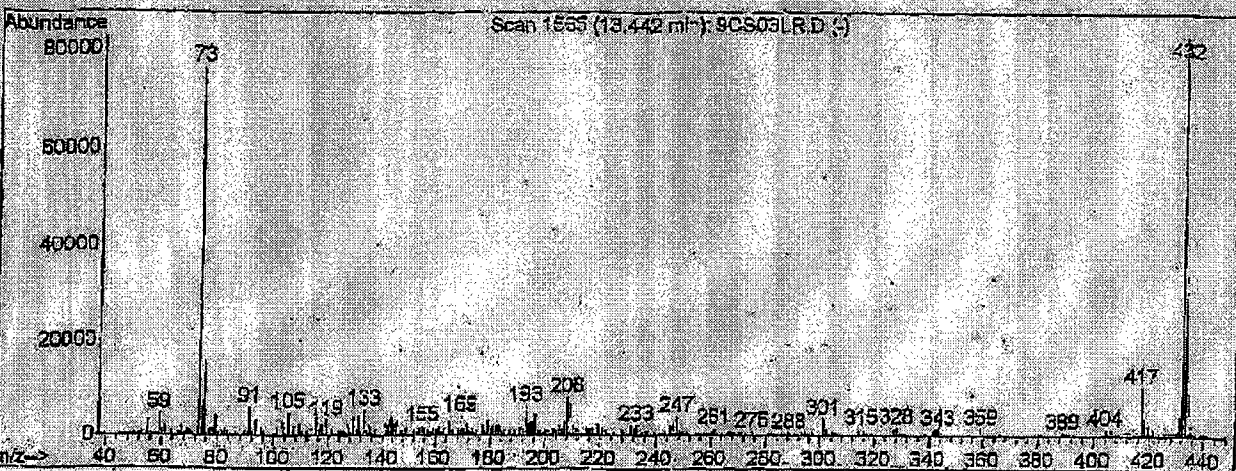
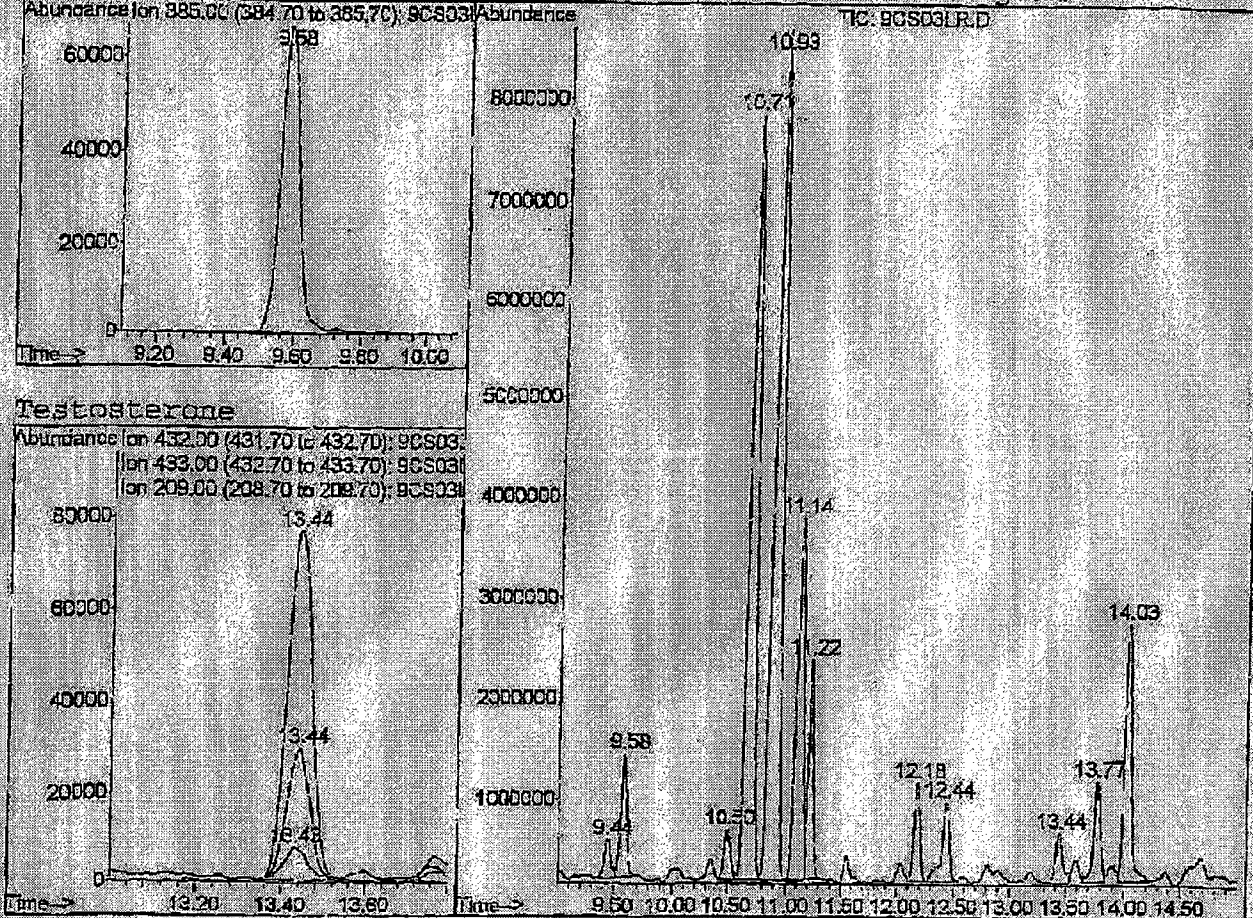
Data File : K:\CHEM\AAS\CONFIRM\MSDA13\9CS03LR.D
Acquired : 29 Jun 6:47 pm
Instrument : MSDA13
Misc Info :
Sample Name: 9CS03 TE A CONFIRMATION LIN

Method File: ANAB97LS
Vial Number: 84

ISTD Ethylmorphine



Total Ion Chromatogram



Handwritten signature and date: 6/30/00

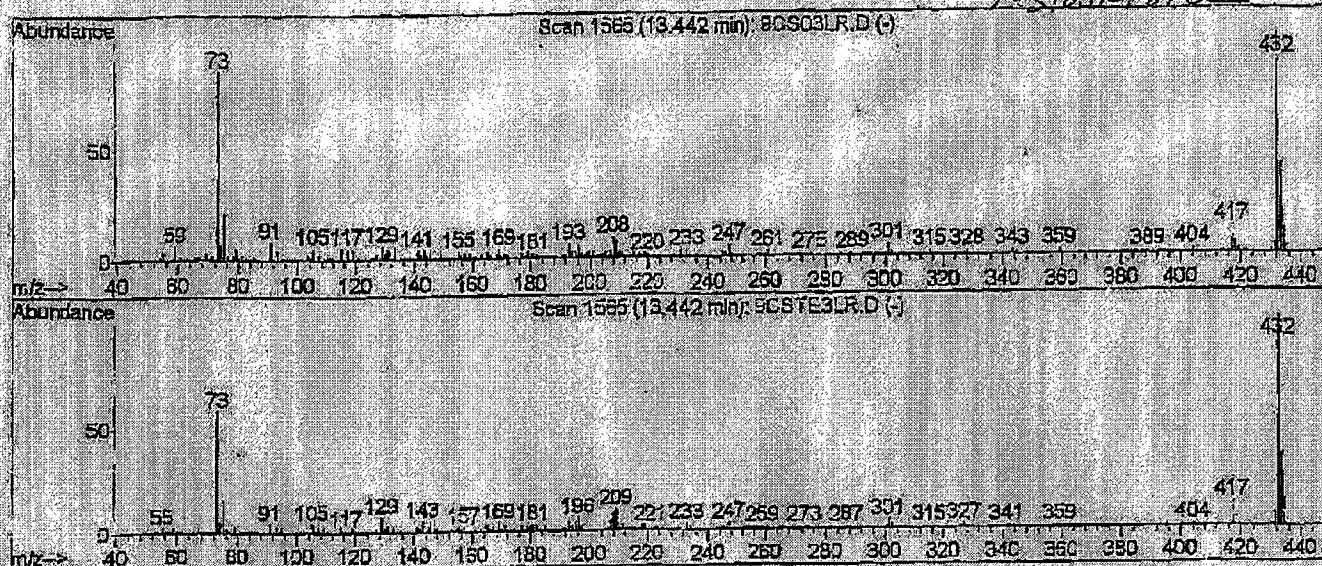
USADA 0039

GDC00524

*** Linear Spectrum Cross Correlation Report ***

>> Cross Correlation for the spectra below = 0.9776 <<

Testosterone



>>> Normalized Tabulation of First Spectrum Printed Above
Result of BIG
Baa

| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 55.05 | 3 | 105.05 | 5 | 169.10 | 4 | 231.10 | 3 |
| 59.05 | 6 | 115.10 | 5 | 171.10 | 2 | 233.20 | 3 |
| 67.10 | 2 | 117.05 | 5 | 176.95 | 2 | 247.20 | 5 |
| 69.10 | 3 | 119.10 | 4 | 179.10 | 3 | 301.10 | 5 |
| 73.10 | 86 | 129.05 | 6 | 193.10 | 6 | 417.30 | 12 |
| 74.10 | 7 | 131.10 | 4 | 194.10 | 3 | 418.20 | 5 |
| 75.10 | 21 | 133.10 | 6 | 195.10 | 3 | 431.35 | 4 |
| 79.00 | 5 | 141.10 | 4 | 196.20 | 5 | 432.25 | 100✓ |
| 81.10 | 2 | 143.10 | 4 | 206.95 | 3 | 433.30 | 41✓ |
| 91.10 | 8 | 155.20 | 3 | 208.10 | 9 | 434.40 | 11 |
| 93.10 | 4 | 165.05 | 3 | 209.10 | 7 | 435.30 | 3 |

>>> Normalized Tabulation of Second Spectrum Printed Above
Result of BIG
Baa

| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|--------|--------|--------|--------|--------|--------|--------|--------|
| 55.10 | 2 | 119.00 | 2 | 180.00 | 2 | 209.20 | 10✓ |
| 59.05 | 2 | 129.00 | 7 | 181.00 | 3 | 233.10 | 3 |
| 67.10 | 2 | 130.10 | 2 | 182.10 | 2 | 247.20 | 3 |
| 73.10 | 58 | 131.10 | 3 | 185.10 | 2 | 301.20 | 4 |
| 74.10 | 5 | 133.00 | 2 | 193.20 | 4 | 417.30 | 12 |
| 75.05 | 15 | 143.00 | 5 | 194.10 | 2 | 418.30 | 4 |
| 79.05 | 3 | 165.10 | 3 | 195.10 | 4 | 431.40 | 6 |
| 91.05 | 4 | 169.10 | 4 | 196.20 | 6 | 432.30 | 100✓ |
| 93.10 | 3 | 171.10 | 3 | 197.20 | 2 | 433.30 | 35✓ |
| 105.10 | 4 | 177.10 | 2 | 207.10 | 4 | 434.40 | 13 |
| 115.10 | 3 | 179.10 | 2 | 208.10 | 8 | 435.30 | 3 |

Musol

UCLA Olympic Analytical Laboratory

'A' CONFIRMATION SUMMARY

Client: USADA
Folder: 7800
UCLA Code: 9CS03

Bottle: [REDACTED]

CONFIRMATION DATA

| Substance: | Testosterone | WADA ISL | | IS = Ethylmorphine | |
|-------------------|--------------|----------|-------|--------------------|---------|
| | Calibrator | Ranges | | NEG QC | 9CS03 |
| GC-MS Datafile | 9CSTE3LR | min | max | 9CSNQCAR | 9CS03LR |
| ISTD RT (min) | 9.60 | 9.50 | 9.70 | 9.60 | 9.58 |
| Compound RT | 13.44 | 13.31 | 13.58 | ND | 13.44 |
| Compound RRT | 1.406 | NA | NA | NA | 1.403 |
| Base peak 432 | 100 | NA | NA | NA | 100 |
| Ion 1 433 | 41 | 33 | 49 | NA | 35 |
| Ion 2 209 | 7 | 2 | 12 | NA | 10 |
| Cross Correlation | NA | NA | NA | NA | 0.9776 |

CONCLUSION:

Sample 9CS03 has a high T/E ratio.

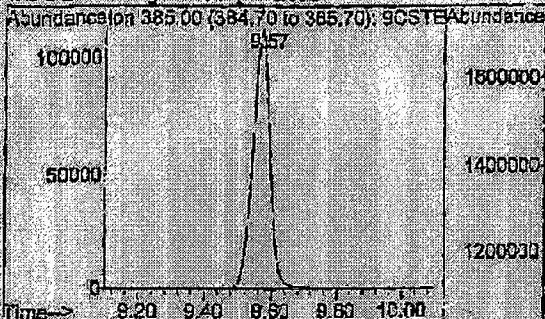
YF 6/30/01 [REDACTED]
no 1/34/01 [REDACTED]

USADA 0041

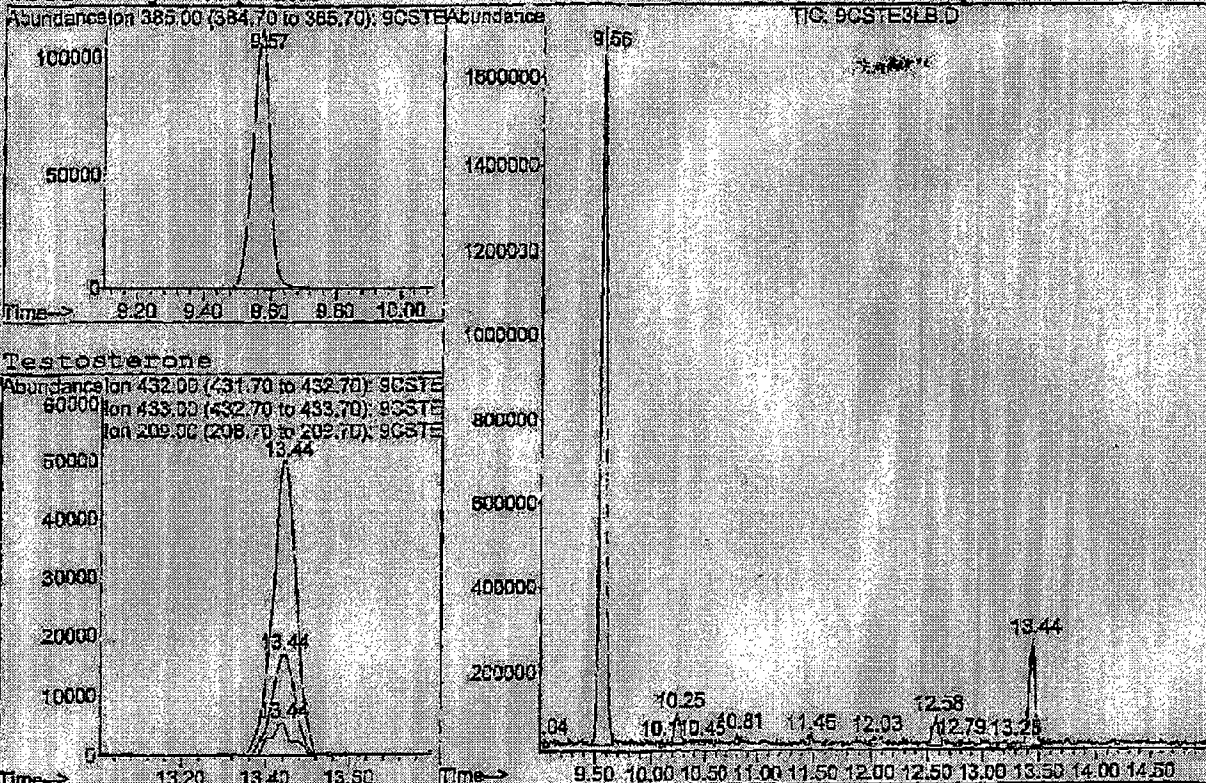
Data File : K:\CHEM\AAS\CONFIRM\MSDA14\9CSTE3LB.D
Acquired : 2 Aug 8:12 pm
Instrument : MSDA14
Misc Info :
Sample Name: TE 4:1 STANDARD LIN

Method File: ANAB97LS
Vial Number: 42

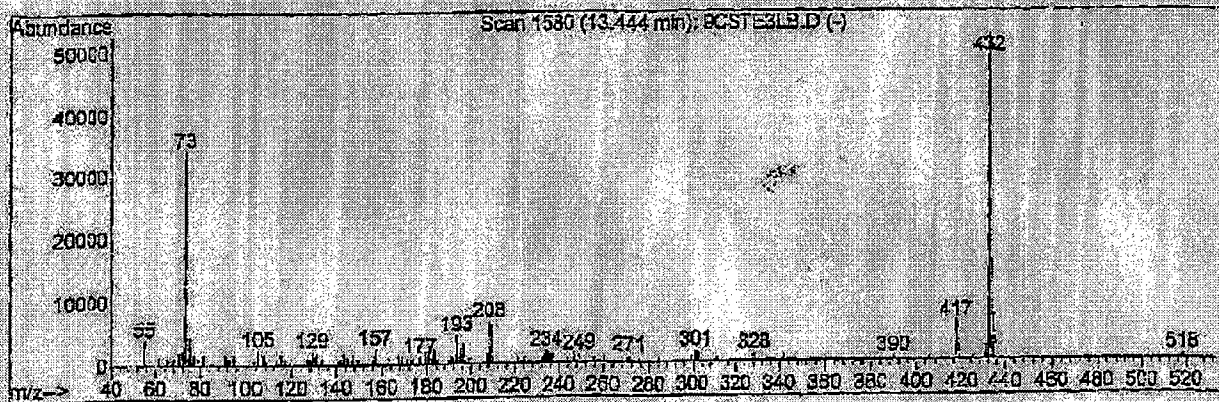
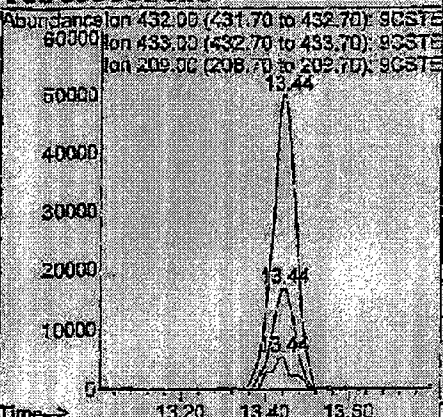
ISTD Ethylmorphine



Total Ion Chromatogram



Testosterone

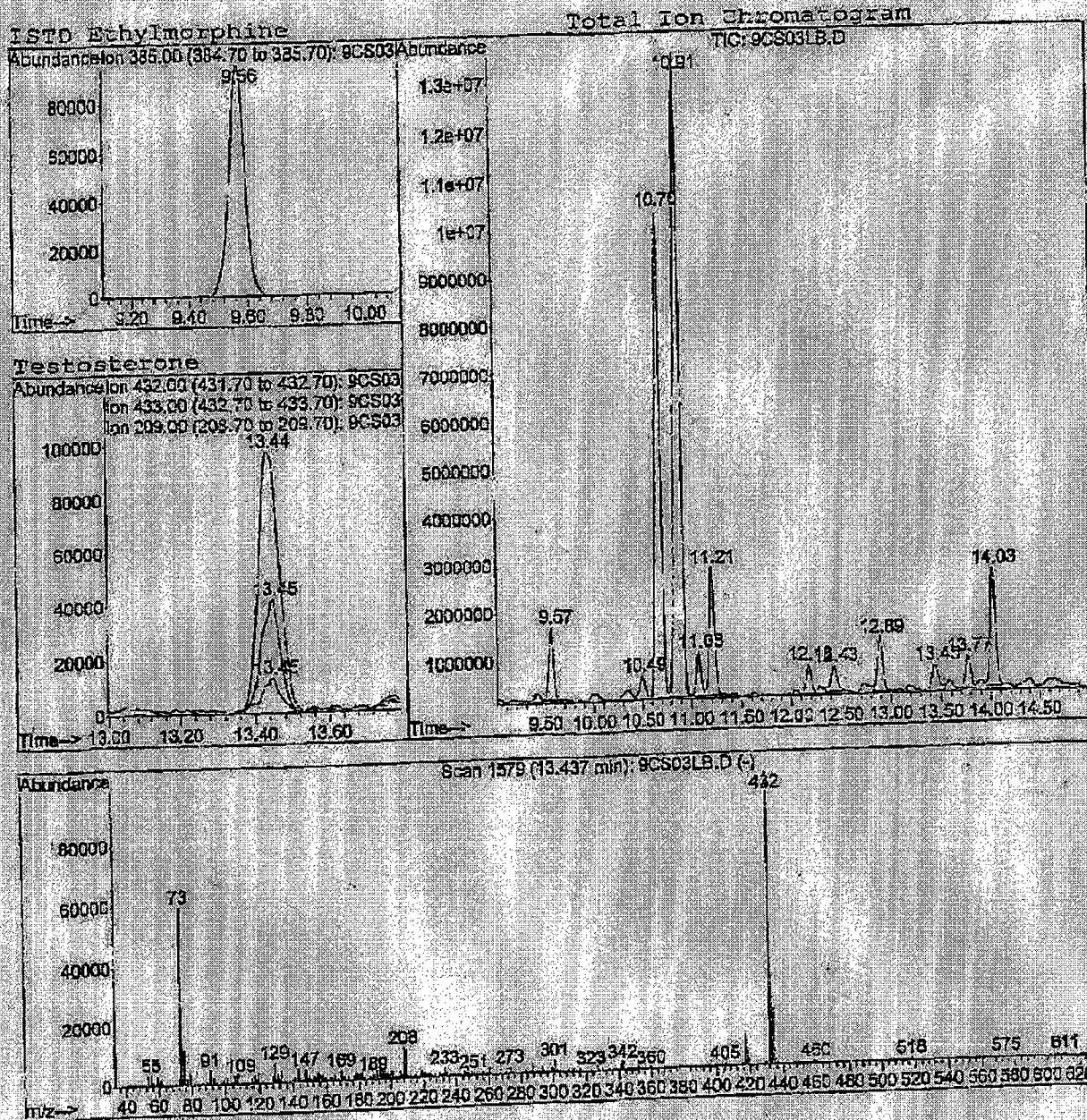


TS 8/31

2/7/10

Date File : K:\CHEM\AAS\CONFIRM\MSDA14\9CS031B.D
 Acquired : 2 Aug 7:20 pm
 Instrument : MSDA14
 Misc Info :
 Sample Name: 9CS03 TE 'B' CONFIRMATION L1N

Method File: ANAB97LS
 Vial Number: 41

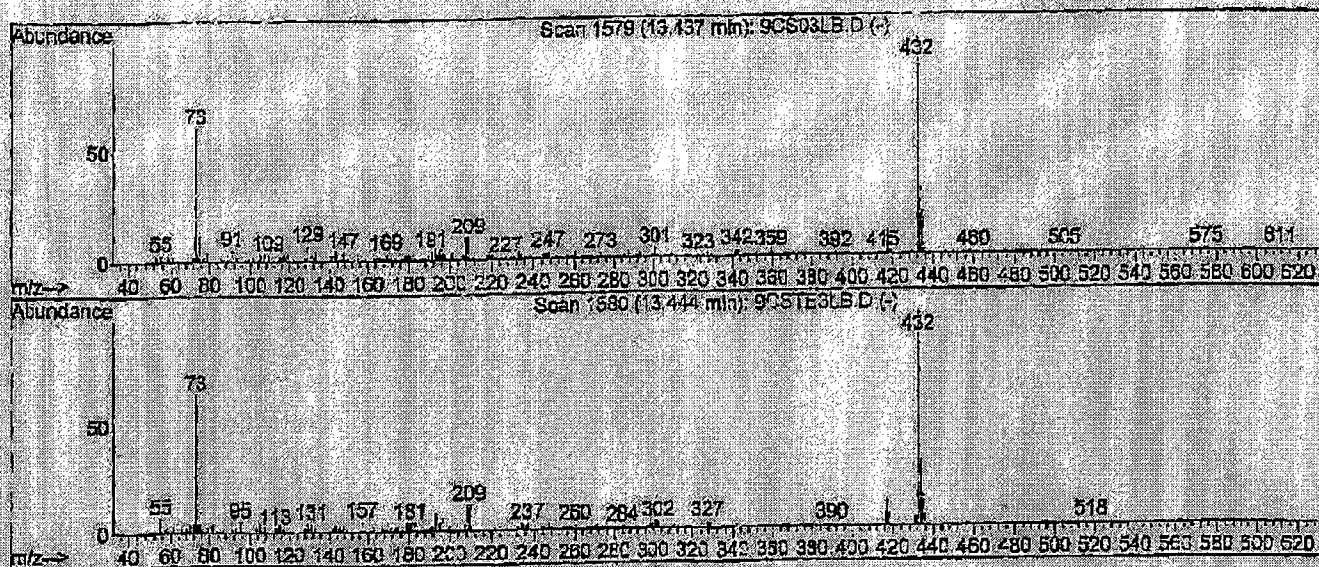


TS 8/31/00
 MS 8/31/00

*** Linear Spectrum Cross Correlation Report ***

p 64

>> Cross Correlation for the spectra below = 0.9787 <<



>>> Normalized Tabulation of First Spectrum Printed Above
Result of BIG

Baa

| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 53.10 | 3 | 105.10 | 3 | 155.10 | 3 | 209.20 | 10 |
| 55.20 | 3 | 107.90 | 3 | 169.15 | 3 | 233.20 | 3 |
| 59.05 | 3 | 109.10 | 3 | 182.15 | 3 | 247.20 | 3 |
| 61.00 | 2 | 117.05 | 3 | 191.30 | 4 | 287.25 | 3 |
| 71.90 | 2 | 119.20 | 3 | 193.20 | 6 | 301.10 | 4 |
| 73.10 | 61 | 129.05 | 3 | 194.10 | 3 | 342.30 | 3 |
| 74.20 | 4 | 131.10 | 3 | 195.20 | 4 | 417.30 | 11 |
| 75.10 | 12 | 132.05 | 2 | 196.10 | 5 | 418.40 | 5 |
| 77.15 | 2 | 133.05 | 4 | 197.10 | 3 | 432.40 | 100 |
| 79.00 | 4 | 143.10 | 5 | 201.10 | 3 | 433.30 | 31 |
| 91.00 | 5 | 147.30 | 4 | 208.05 | 10 | 434.40 | 20 |

>>> Normalized Tabulation of Second Spectrum Printed Above
Result of BIG

Baa

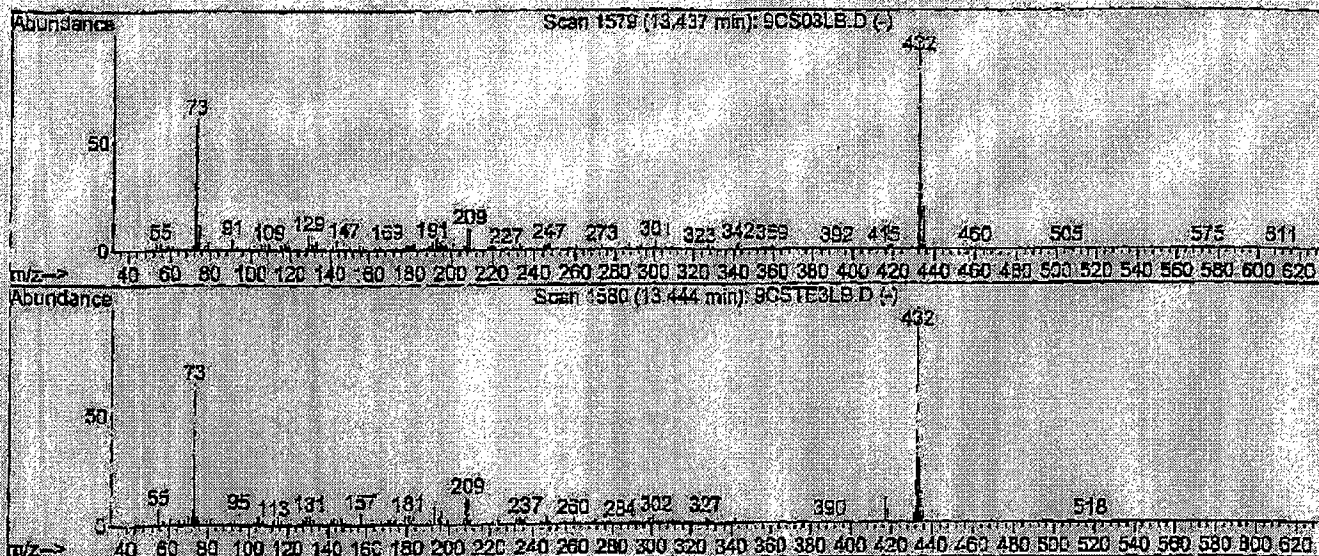
| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 55.10 | 7 | 105.00 | 4 | 160.80 | 4 | 246.80 | 3 |
| 61.20 | 3 | 107.10 | 3 | 183.10 | 4 | 301.10 | 3 |
| 68.00 | 2 | 115.05 | 4 | 193.30 | 8 | 302.50 | 3 |
| 70.20 | 4 | 128.10 | 2 | 195.10 | 4 | 327.30 | 3 |
| 72.00 | 4 | 129.10 | 4 | 196.20 | 5 | 417.20 | 11 |
| 73.10 | 55 | 130.00 | 2 | 199.20 | 2 | 418.30 | 6 |
| 74.10 | 5 | 131.10 | 4 | 207.20 | 3 | 431.20 | 4 |
| 75.10 | 4 | 143.10 | 3 | 208.20 | 12 | 432.40 | 100 |
| 81.20 | 3 | 147.20 | 3 | 209.20 | 12 | 433.40 | 30 |
| 92.80 | 2 | 157.10 | 5 | 234.20 | 3 | 434.40 | 13 |
| 95.10 | 5 | 179.10 | 4 | 237.10 | 3 | 435.30 | 5 |

TS 8/31

ms 9/7/10

USADA 0067

>> Cross Correlation for the spectra below = 0.9787 <<



>>> Normalized Tabulation of First Spectrum Printed Above

Result of SIG

Baa

| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 53.10 | 3 | 105.10 | 3 | 155.10 | 3 | 209.20 | 10 |
| 55.20 | 3 | 107.00 | 3 | 169.15 | 3 | 233.20 | 3 |
| 59.05 | 3 | 109.10 | 3 | 182.15 | 3 | 247.20 | 3 |
| 61.00 | 2 | 117.05 | 3 | 191.30 | 4 | 287.25 | 3 |
| 71.90 | 2 | 119.20 | 3 | 193.20 | 6 | 301.10 | 4 |
| 73.10 | 61 | 129.05 | 7 | 194.10 | 3 | 342.30 | 3 |
| 74.20 | 4 | 131.10 | 3 | 195.20 | 4 | 417.30 | 11 |
| 75.10 | 12 | 132.05 | 2 | 196.10 | 5 | 418.40 | 5 |
| 77.15 | 2 | 133.05 | 4 | 197.10 | 3 | 432.40 | 100 |
| 79.00 | 4 | 143.10 | 5 | 201.10 | 3 | 433.30 | 31 |
| 91.00 | 5 | 147.30 | 4 | 208.05 | 10 | 434.40 | 20 |

>>> Normalized Tabulation of Second Spectrum Printed Above

Result of SIG

Baa

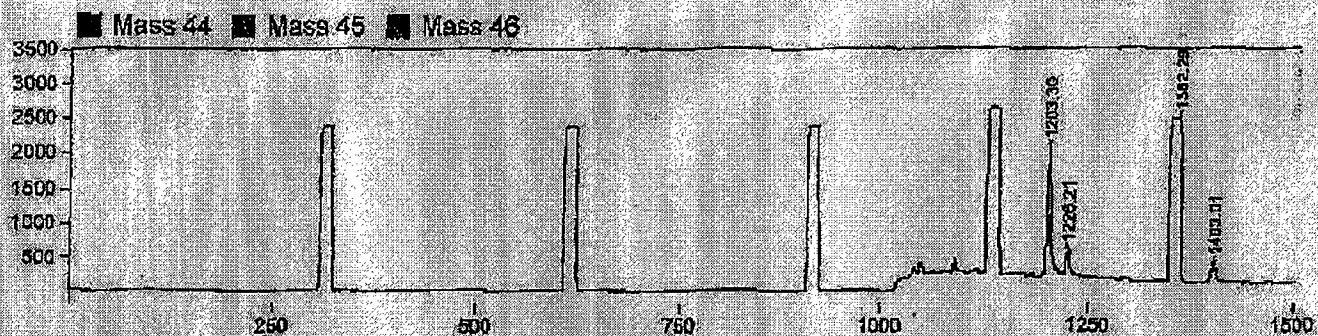
| m/z | abund. | m/z | abund. | m/z | abund. | m/z | abund. |
|-------|--------|--------|--------|--------|--------|--------|--------|
| 55.10 | 7 | 105.00 | 4 | 180.80 | 4 | 246.80 | 3 |
| 61.20 | 3 | 107.10 | 3 | 183.10 | 4 | 301.10 | 3 |
| 68.60 | 2 | 115.05 | 4 | 193.30 | 8 | 302.50 | 3 |
| 70.20 | 4 | 128.10 | 2 | 195.10 | 4 | 327.30 | 3 |
| 72.00 | 4 | 129.10 | 4 | 196.20 | 6 | 417.20 | 11 |
| 73.10 | 65 | 130.00 | 2 | 199.20 | 2 | 418.30 | 6 |
| 74.10 | 5 | 131.10 | 4 | 207.20 | 3 | 431.20 | 4 |
| 75.13 | 4 | 143.10 | 3 | 208.20 | 12 | 432.40 | 100 |
| 81.20 | 3 | 147.20 | 3 | 209.20 | 12 | 433.40 | 30 |
| 92.80 | 2 | 157.10 | 5 | 234.20 | 3 | 434.40 | 14 |
| 95.10 | 5 | 179.10 | 4 | 237.10 | 3 | 435.30 | 5 |

TS 8/31

ms 8/7/10

UCLA Olympic Analytical Laboratory

| AS | AS Method | Identifier | Comment | Preparation | Post Script | Method |
|----|-----------|----------------|---------|-------------|-------------|-------------------|
| X | 52 | >Internal No 9 | 9CS | 10 mL | | method1[dols] met |



| Rt [s] | d 13C/12C [per mil] vs. VPDB |
|--------|------------------------------|
| 1203.3 | -32.736 |
| 1226.2 | -31.737 |
| 1403.0 | -26.417 |

DEUTSCHE SPORTHOCHSCHULE
KÖLN
INSTITUT FÜR BIOCHEMIE
IOC AKKREDITIERTES LABOR
FÜR DOPINGANALYTIK

Prof. Dr. Wilhelm Schänzer * Carl-Diem-Weg 6 * 50933 Köln

Prof. Dr. Wilhelm Schänzer
C/O Deutsche Sporthochschule Köln
INSTITUT FÜR BIOCHEMIE
Carl-Diem-Weg 6
50933 Köln

TELEFON: 0221-4971313
TELEFAX: 0221-4973236

total pages -1-

KÖLN, DEN 8. April 2003

Documentation Analysis Report 202/03

Code: 188702

Decision criterias for GC/C/IRMS results

Based on our reference values the following criterias indicate an application of testosterone or testosterone prohormones:

Difference of the $\delta^{13}\text{C}$ [‰]- values between androsterone and pregnanediol > 2.6 or
difference between the $\delta^{13}\text{C}$ [‰]- values of etiocholanolone and pregnanediol > 3.3


Prof. Dr. Wilhelm Schänzer

| | | | | |
|----|-------|--------|-----|-----|
| | NH | DS/751 | PR | SG |
| S1 | IN | 8/4 | TEC | MED |
| S2 | OUT | | TH | VE |
| S3 | INDEX | | REC | AP |

GC/C/IRMS - Results

26.02.03

Federation:

Event:

Code-Nr.:

188702

Lab-Nr.:

1013/03 A

Sample receipt:

11.02.03

Results:

The ratio testosterone/epitestosterone is higher than 6 (25.3 \pm 0.31, n=3); the GC/C/IRMS results indicate an application of testosterone or testosterone prohormones

For the sample with the code number 188702 the following $\delta^{13}\text{C}$ [‰]- values were obtained:

testosterone metabolites:

etiocholanolone - 30.5 \pm 0.2 (n=5)

androsterone - 29.5 \pm 0.3 (n=5)

internal reference compounds:

11 β -hydroxy-androsterone - 20.46; - 20.17

pregnanediol - 21.1 \pm 0.1 (n=3)

Conclusions

The $\delta^{13}\text{C}$ [‰]-values of the testosterone metabolites indicate the application of testosterone or testosterone prohormones.

CONFIDENTIAL
Carbon Isotope Ratio Report

August 4, 2003

Terry Madden
The United States Anti-Doping Agency
2550 Tenderfoot Hill Street, Suite 200
Colorado Springs, CO 80906
Fax 719-785-2028

Specimen number: [REDACTED]
USADA Site ID: OOC
UCLA Code: [REDACTED]

Analysis: "Diol" assay using isotope ratio mass spectrometry (see letter of June 2001 for criteria and assay details).

Result: Indeterminate, we suggest obtaining additional samples.

Analytical data:

| | <u>5β-adiol</u> | <u>5α-adiol</u> | <u>5β-pdol</u> |
|----------------|----------------------------------|-----------------------------------|---------------------------------|
| Sample ZE809-A | -23.6 | -27.7 | -23.3 |


Don H. Catlin, M.D. **AUG 04 2003**
Date



WADA Accredited

UCLA Olympic Analytical Laboratory
UCLA School of Medicine
2122 Granville Ave Los Angeles CA 90025
Phone (310) 825-2635 FAX (310) 206-9077



ISO/IEC 17025
Biological Testing
Certificate: 1420-01

CONFIDENTIAL
Drug Testing Report USADA [REDACTED]

July 10, 2006

Terrence Madden
The United States Anti-Doping Agency
2550 Tenderfoot Hill Street, Suite 200
Colorado Springs, CO 80906
Fax 719-785-2028

Specimen number: [REDACTED]
USADA Site ID: OOC
UCLA Code: [REDACTED]
Sport and Event: Cycling OOC
Collection Date: 05/28/06
Date Received at Lab: 05/31/06

Analysis: The urine sample was analyzed for anabolic steroids and masking agents, diuretics, and hCG using methods 1001, 2001, and 6001. The sample was also analyzed by the "Diol" assay using method 8001, steroids by carbon isotope ratio by GC/IRMS (see letter of June 2001 for criteria and assay details).

| Analytical data: | 5 β -adiol | 5 α -adiol | 5 β -pdol |
|------------------|------------------|-------------------|-----------------|
| | -32.7 | -31.7 | -25.4 |

Unit of measurement for the three values is $\delta^{13}\text{C}$ [‰]

GCMS Laboratory conclusion: The TE ratio is greater than four. The results for the other testing methods listed above are negative.


Michael Sekera
Certifying Scientist

cc: World Anti-Doping Agency
International Cycling Union

This report shall not be reproduced, except in full, without the written approval of the laboratory.

Page 1 of 1

Shah

June 21, 2001

To: Clients of the UCLA Olympic Laboratory

Regarding: Carbon Isotope Ratio measurements, Update 3

This letter is to update you on our carbon isotope ratio method and to explain the wording we use in our current reports.

DIOL ASSAY

Requests: Currently when you request a CIR analysis we perform the "Diol" assay. The procedure is to request a CIR analysis by fax or email and to provide the sample number. We will check the original data on the sample and determine if the analysis is likely to be successful. If not, we will advise you that we do not believe the analysis will be successful and we may suggest an alternative approach. In order to enhance our understanding of the analysis we also ask that you give us all sample numbers of prior samples from the same athlete that were analyzed at UCLA.

The "Diol" assay determines the carbon isotope ratio (delta value) of two diol metabolites of testosterone which we refer to as M1 and M2, and one metabolite of a testosterone precursor (Pdiol). [See the metabolic map attached.] The assay determines the ratio of $^{13}\text{C}/^{12}\text{C}$ for each of these three steroids. The units are usually called "delta units". In addition to the delta values for these steroids, two other types of measurements are calculated. The first is the ratio of the metabolites to the precursor. Two ratios are calculated: M1/Pdiol and M2/Pdiol. The second is the difference between the metabolites and the Pdiol: M1 Pdiol and M2-Pdiol.

Endogenous reference compound: The Pdiol serves as an endogenous reference compound (ERC). Since it is a metabolite of a precursor (see map) in the testosterone metabolic scheme, its delta value does not change when testosterone is administered. In the typical positive case, the delta values of M1 and M2 are low and the delta value of Pdiol is within the normal range. In negative cases, all three diols have similar delta values. The reporting terminology will be: Positive, Negative, or Indeterminate.

A **POSITIVE** report means that the delta values for both M1 and M2 are at least three standard deviation (SD) units less than the mean (average) of a group of 73 normal males, and the delta value for Pdiol is within 3 SD of the mean of normal males. In addition the two ratios (M1/Pdiol and M2/Pdiol) and the two differences (M1-Pdiol and M2-Pdiol) are more than 3 SD from the range of normal values. These criteria are very conservative because all must be met for the sample to be declared positive.

A **NEGATIVE** report means that all three delta values, the two difference scores, and the two ratios are within the normal range.

An **INDETERMINATE** report means that we were not able to obtain definitive data. The most common reasons for this are insufficient sample volume and low concentrations of the steroids. Since there are several criteria for a positive report, it is also possible that a sample will have one ratio and one difference score that are normal and the other ratio and difference score will be abnormal. Other combinations of results are possible. Based on our current understanding of the theory underlying CIR measurements, we believe that in these mixed cases that we classify as indeterminate, some of the molecules of M1 or M2 are derived from pharmaceutical testosterone (or another exogenous steroid which is metabolized to

testosterone) and some of the molecules are from natural (endogenous) testosterone. This is expected when the body contains a mixture of pharmaceutical and natural testosterone and metabolites. This situation is likely to occur at the later times in the curve that relates delta values to time since drug administration. As our clients gain further understanding of the CIR analysis, you may have other opinions about how we report mixed cases.

SAMPLES FROM OTHER LABORATORIES

Occasionally we are asked to perform a CIR analysis on a sample that was originally processed at another sample. We are not comfortable with this for four reasons: 1) we do not know the details of handling and storage of the sample in the other laboratory, 2) we do not have any control over the chain-of-custody until the sample arrives at UCLA, 3) we do not have screening data (T/E, testosterone concentration, etc.) obtained at UCLA therefore we cannot adequately plan the analysis, and 4) we do not have control samples that were handled in the same way. We do not have any reason to believe that the CIR analysis is affected by storage conditions, time factors, or temperature nevertheless we cannot be absolutely certain that our in-house control data applies to such samples. In the future we will decline to perform the CIR analysis unless there is sufficient sample volume to perform both a steroid analysis and a CIR analysis.

ANDRO & ETIO ASSAY

Under certain circumstances we also offer the Andro & Etio assay. The advantages are that it is simpler to perform, less expensive, and it almost always yields definitive results. The disadvantage is that there is no PdIOL to serve as an ERC, therefore we are not able to calculate ratios or difference scores. Most clients seem to prefer the DIOL assay, however we suspect that in the future the Andro&Etio assay will be developed to the point where the lack of an ERC will not be a disadvantage. At this time we recommend the "diol" assay. Occasionally we will advise you that it is not possible to perform the DIOL assay and that the only CIR option is the Andro & Etio assay.

GENDER

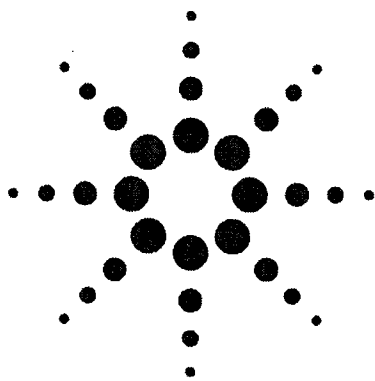
At the present time we do not recommend performing the CIR analysis on samples from females. If you still wish to have the analysis we will determine if it is feasible to proceed based on the existing data from the athlete.

EPITESTOSTERONE

We have been working on a CIR assay for epitestosterone and we have recently presented our findings at a national meeting. There is no peer reviewed publication at this time and it will take about a year to prepare a manuscript and get it published. Nevertheless, at your request we will conduct the epitestosterone analysis on urine samples reported "epitestosterone > 200 ng/ml".

TURN-AROUND-TIME

In an attempt to keep the costs down we are performing the analyses one week per month. Typically this is the fourth week of the month. It takes about a week to complete the analysis, calculate the results, and the issue the report.



Agilent Gas Chromatographs

Fundamentals of Gas Chromatography



Agilent Technologies

Notices

© Agilent Technologies, Inc. 2002

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

Manual Part Number

G1176-90000

Edition

First edition, May 2002

Printed in USA

Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, Delaware 19808-1610 USA

In This Book...

This book contains information for using your gas chromatograph (GC) effectively.

1 What Gas Chromatography Is

This chapter describes gas chromatography—its effects and its uses—and the chromatographic hardware.

2 Injecting Samples

This chapter describes the most common ways of getting the sample into the GC.

3 Separating Components

The column separates the sample into components. This chapter tells how this works and how to use it.

4 Detecting Components

This chapter describes three common GC detectors.

5 Interpreting Chromatograms

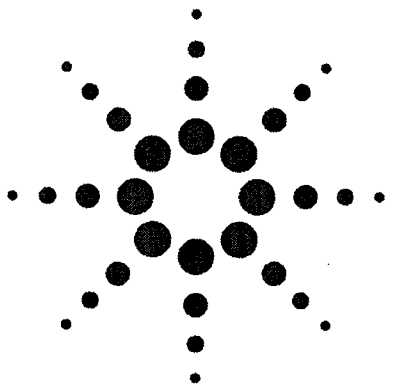
The final chapter discusses how to identify peaks and how to determine the amounts of each component.

Contents

| | | |
|----------|--|----|
| 1 | What Gas Chromatography Is | |
| | A Separation in Time | 10 |
| | The System | 11 |
| | The gas source | 12 |
| | The inlet | 12 |
| | The column | 14 |
| | The detector | 14 |
| | Data reduction | 15 |
| | Instrument control | 15 |
| 2 | Injecting Samples | |
| | Injection Ports | 18 |
| | Packed port | 18 |
| | Split/Splitless port | 19 |
| | Injection technique | 22 |
| | Benefits of automatic injection | 23 |
| | Valves | 24 |
| | Gas sampling | 24 |
| | Liquid sampling | 24 |
| | Inlet Temperature | 25 |
| | Gas samples | 25 |
| | Liquid samples | 25 |
| 3 | Separating Components | |
| | How a Column Separates Components | 28 |
| | The basic principles of chromatography | 29 |

| | |
|-------------------------------------|----|
| Column Types | 30 |
| Capillary columns | 30 |
| Packed columns | 30 |
| Column tubing | 31 |
| Column Characteristics | 32 |
| Column efficiency | 32 |
| Gas control | 33 |
| Column resolution | 33 |
| Column selectivity | 34 |
| Capillary or Packed? | 35 |
| Column Temperature | 36 |
| Isothermal oven | 36 |
| Programmed oven | 37 |
| 4 Detecting Components | |
| Thermal Conductivity (TCD) | 40 |
| How it works | 40 |
| Flame Ionization (FID) | 42 |
| How it works | 42 |
| Electron Capture (ECD) | 43 |
| How it works | 43 |
| 5 Interpreting Chromatograms | |
| Peak Measurements | 47 |
| Retention time | 47 |
| Peak size | 47 |
| Integrators and data systems | 48 |
| Component Identity | 49 |
| Component Amount | 50 |
| Uncalibrated calculations | 50 |

| | |
|-------------------------|----|
| Area and height percent | 50 |
| Calibrated calculations | 52 |
| Normalization | 54 |
| External standard | 55 |
| Internal standard | 56 |



**Agilent Gas Chromatographs
Fundamentals of Gas Chromatography**

1 What Gas Chromatography Is

| | |
|----------------------|----|
| A Separation in Time | 10 |
| The System | 11 |
| The gas source | 12 |
| The inlet | 12 |
| The column | 14 |
| The detector | 14 |
| Data reduction | 15 |
| Instrument control | 15 |

Gas chromatography (GC) is a laboratory technique that separates mixtures into individual components. It is used to identify components and to measure their concentrations.



1 What Gas Chromatography Is

A Separation in Time

Rather than a physical separation (such as distillation and similar techniques), GC creates a time separation.

It does this by passing the vaporized mixture (or a gas) through a tube containing a material that retards some components more than others. This separates the components in time. After detection, the result is a chromatogram (Figure 1), where each peak represents a different component of the original mixture.

The appearance time can be used to identify each component; the peak size (height or area) is a measure of the amount.

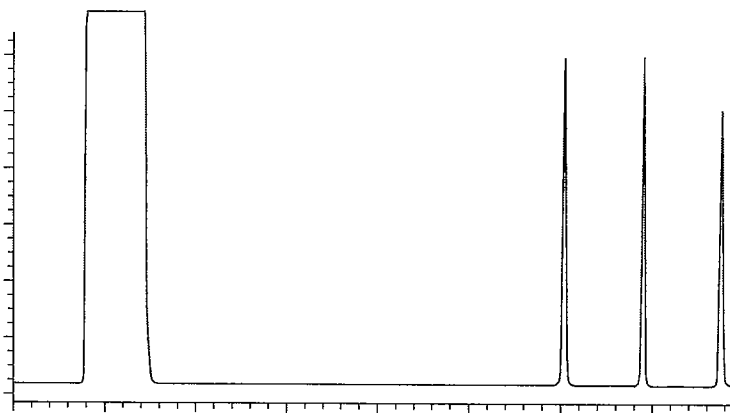


Figure 1 A typical chromatogram

The System

A gas chromatographic system consists of:

- A regulated and purified carrier gas source, which moves the sample through the GC
- An inlet, which also acts as a vaporizer for liquid samples
- A column, in which the time separation occurs
- A detector, which responds to the components as they occur by changing its electrical output
- Data interpretation of some sort

This is summarized in Figure 2.

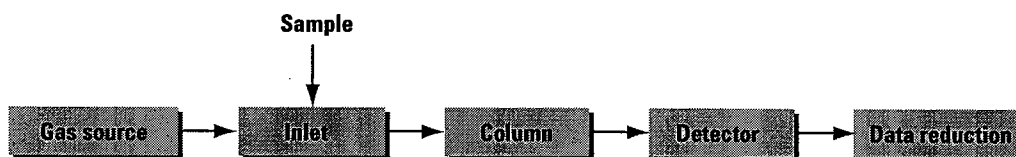


Figure 2 A chromatographic system

The gas source

The carrier gas must be pure. Contaminants may react with the sample or the column, create spurious peaks, load the detector and raise baselines, and so on. A high-purity gas with traps for water, hydrocarbons and oxygen is recommended. See Figure 3.

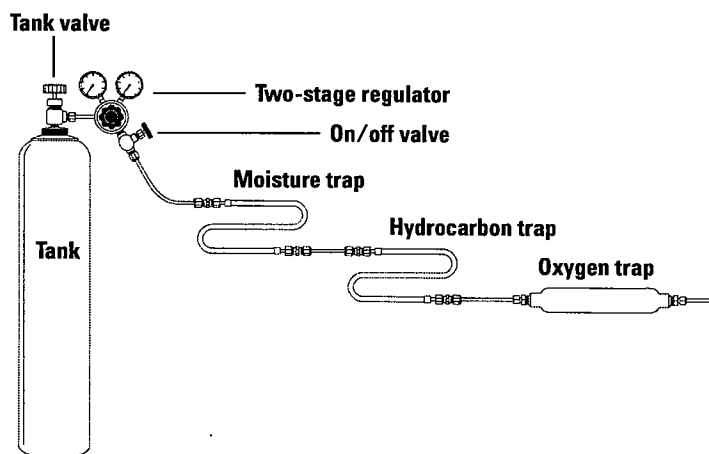


Figure 3 The gas source.

When a house gas supply, rather than separate tanks, is used, have traps for each GC and place them as close to the back of the instrument as possible.

The inlet

The inlet introduces the vaporized sample into the carrier gas stream. The most common inlets are injection ports and sampling valves.

Injection ports

Handle gas or liquid samples. Often heated to vaporize liquid samples. Liquid or gas syringes are used to insert the sample through a septum into the carrier gas stream. The principle (not a real design) is shown in Figure 4.

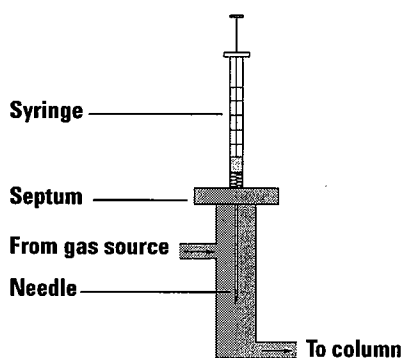


Figure 4 Injection port

Sampling valves

The sample is flushed from a loop which is mechanically inserted into the carrier gas stream. Different valves are used for liquids and gases, because sample volumes are usually quite different. The principle (not a real design) is shown in Figure 5.

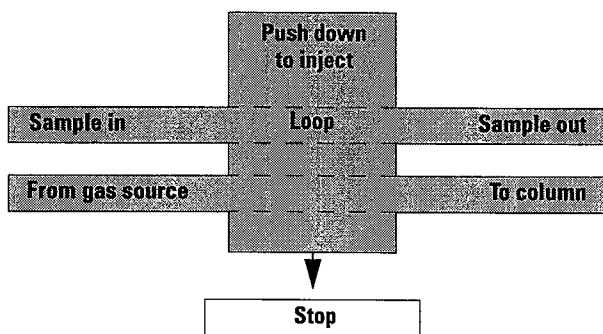


Figure 5 Sampling valve

1 What Gas Chromatography Is

Sample valves are often connected to an inlet, especially the split/splitless inlet in the split mode.

The column

The separation happens here. Because the column type is selected by the user, many different analyses can be performed using the same equipment.

Most separations are highly temperature-dependent, so the column is placed in a well-controlled oven. See Figure 6.

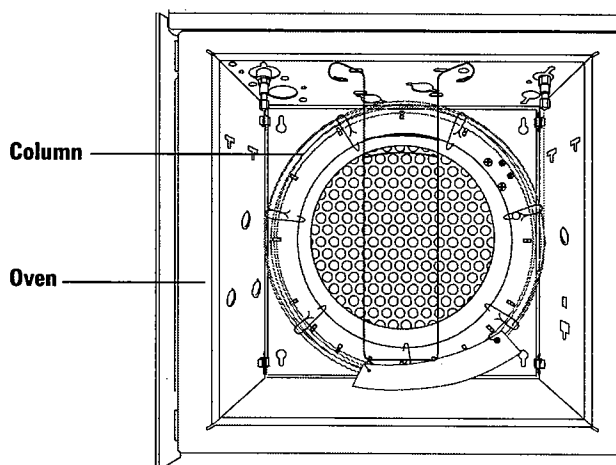


Figure 6 The column and oven

The detector

The gas stream from the column, which contains the separated components, passes through a detector. The output from the detector becomes the chromatogram. See Figure 7.

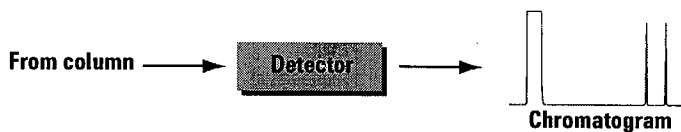


Figure 7 The detector

Several detector types are available, but all perform the same tasks:

- Produce a stable electrical signal (the baseline) when pure carrier gas (no components) is in the detector.
- Produce a different signal when a component is passing through the detector.

Data reduction

Measurement

The chromatogram leaves the detector as an electrical signal. It can be:

- Recorded on a strip chart recorder
- Processed by a digital integrator
- Processed by a computer-based data system

A strip chart recording must be measured to determine the peak times and sizes. Integrators and data systems perform these measurements directly. They are strongly recommended because of their reproducibility and sensitivity.

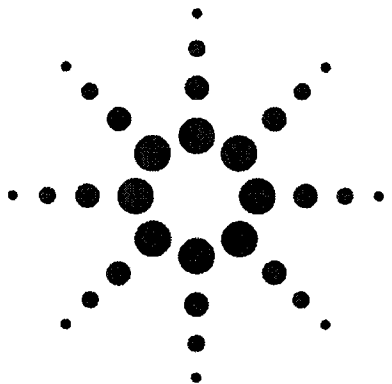
Calculation

The list of times and sizes must be converted to component names and amounts. This is done by comparison to times and responses of known samples (calibration samples). This can be done manually, but for speed and accuracy, a data system is best.

Instrument control

Some data system/GC combinations also provide direct control of the GC by the data system computer. This allows the creation of stored methods, which are invoked as needed, and permits a high degree of analysis automation.

1 What Gas Chromatography Is



2 Injecting Samples

| | |
|---------------------------------|----|
| Injection Ports | 18 |
| Packed port | 18 |
| Split/Splitless port | 19 |
| Injection technique | 22 |
| Benefits of automatic injection | 23 |
| Valves | 24 |
| Gas sampling | 24 |
| Liquid sampling | 24 |
| Inlet Temperature | 25 |
| Gas samples | 25 |
| Liquid samples | 25 |

Some samples are already gases (such as room or outside air, heating gas, etc.) and can be injected directly using either a gas syringe or a gas sampling valve.

Most samples are liquids and must be vaporized in order to be analyzed by gas chromatography. This is usually done with a heated injection port in combination with either a liquid syringe or liquid sampling valve.



Injection Ports

The design and choice of injection ports depends on the column diameter and type. The column types, packed and capillary, are described in the next chapter.

Packed columns and wide-bore capillary columns use the packed port; narrow-bore capillary columns use the split/splitless port.

Packed port

The packed port was developed for packed columns. Removable liners adapt it for the specific column diameter, usually either 1/8- or 1/4-inch. A typical design is shown in Figure 8.

When wide-bore capillary columns appeared, liners were created to allow their use with the packed port. These columns have sample capacities similar to packed columns.

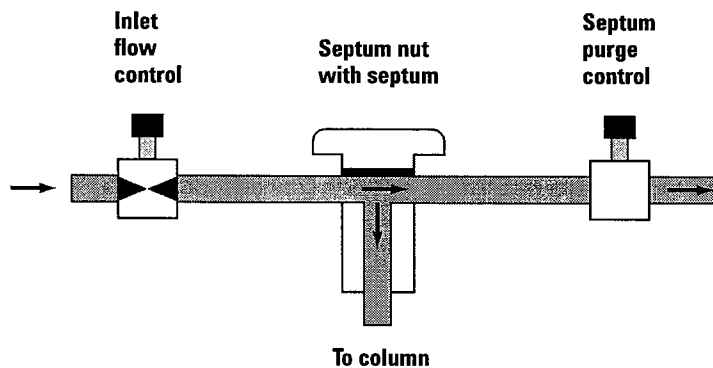


Figure 8 Packed injection port

The sample is injected with a syringe inserted through the septum into the carrier gas stream. The heated port vaporizes it (if it is a liquid) and the carrier gas sweeps it into the column.

Split/Splitless port

The split/splitless port, used with capillary columns, has two operating modes.

Split mode

Capillary columns have low sample capacities. Very small sample sizes, usually much less than a microliter, must be used to avoid overloading the column.

It is very difficult to handle such small sample sizes. The split mode provides a way to inject a normal-size sample, vaporize it, and then transfer only part of it to the column for analysis. The rest is vented to waste.

A typical split/splitless port in *split mode* is shown in Figure 9.

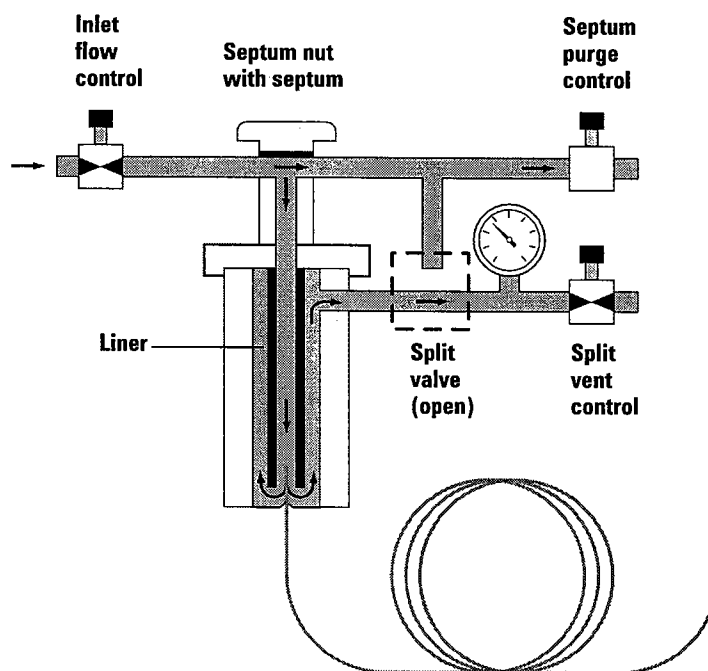


Figure 9 Split mode

2 Injecting Samples

The split valve is open and remains that way. The sample is injected into the liner, where it vaporizes. The vaporized sample divides between the column (high flow resistance) and the split vent (adjustable flow resistance).

Splitless mode

This mode is particularly well suited to low concentration samples. It traps the sample at the head of the column while venting residual solvent vapor in the inlet to waste.

Two steps are involved:

1 Sample injection

Close the split valve. The carrier flow divides between the septum purge and the column. The pressure at the head of the column, and therefore the flow through it, is set by the split vent control.

Inject the sample. The solvent, the major component, creates a saturated zone at the head of the column which traps the sample components.

Figure 10 shows the flows at injection in the *splitless* mode.

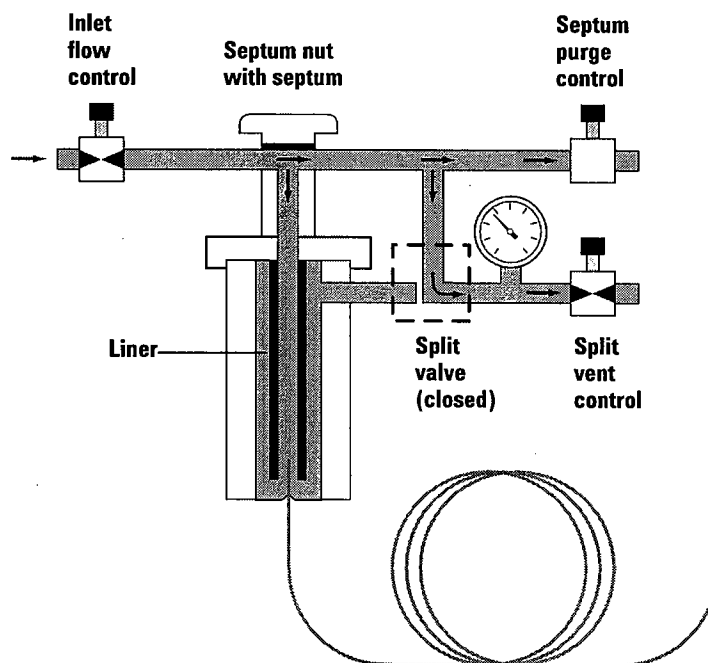


Figure 10 Splitless mode at injection

2 Inlet purge

After the sample has been trapped on the column, open the split valve. The residual vapor in the inlet, now mostly solvent, is swept out the vent.

The flows are now the same as in the split mode (Figure 9).

Raise the oven temperature to begin moving the components through the column.

This works well for components with boiling points higher than that of the solvent. The solvent peak will be large. The oven temperature profile is used to separate peaks of interest from the solvent.

Splitless mode steps

A successful splitless injection consists of these steps:

- 1 Vaporize the sample and solvent in a heated inlet.
- 2 Use a low column temperature to create a solvent-saturated zone at the head of the column.
- 3 Use this zone to trap and reconstitute the sample at the head of the column.
- 4 After all, or at least most, of the sample has transferred to the column, discard the remaining vapor in the inlet by opening the split vent valve.
- 5 Raise the oven temperature to release the solvent and then the sample from the head of the column.

Starting values

You must experiment to find the optimum parameters. Table 1 contains some suggested starting values:

Table 1 Splitless mode inlet parameters

| Parameter | Suggested starting value |
|----------------------------|-------------------------------------|
| Oven temperature | 10°C below solvent boiling point |
| Oven initial time | ≥ Split vent valve open time |
| Split vent valve open time | Liner volume x 2 / Column flow rate |

Injection technique

Each peak begins as part of a region of vaporized sample surrounded by carrier gas. This width of this region broadens by diffusion while the peaks are in the column. No peak can be narrower than the initial region.

Since it is much easier to separate narrow peaks than broad ones, the width of the initial region must be minimized. The ideal injection is:

- 1 Fill the syringe and adjust the amount.
- 2 Push the needle through the injection port septum as far as it will go (port designers assume that you will do this).
- 3 Press the syringe plunger quickly.
- 4 Immediately withdraw the needle from the port.

The important parameter is speed. Any hesitation leads to increased region width.

A skilled operator can achieve 3 to 4% repeatability in sample size, provided he uses the technique described. Mechanical devices that limit the syringe plunger travel can improve on this.

Avoid techniques in which the sample is trapped between two air bubbles. This requires you to make two estimates and doubles the error in sample size.

Benefits of automatic injection

Automatic injectors provide a solution to the injection problem. They make highly reproducible injections. Because of this, they often permit a simpler calculation of peak amount (external instead of internal standard).

If part of an automatic sampler (equipped with a sample tray and connected to a data system), fully automated analyses become possible.

Valves

Gas sampling

A gas sampling valve consists of a sample loop and a means of shifting it in and out of the carrier gas stream.

A common form of the mechanism is shown in Figure 11.

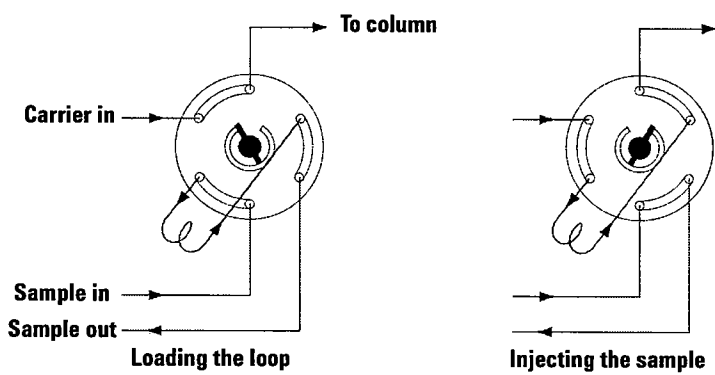


Figure 11 Gas sampling valve

Sample size is determined by the sample loop. This is replaceable, so that a single valve can provide a variety of highly reproducible sample injection sizes.

Liquid sampling

The principle is the same as for a gas sampling valve. Because a liquid sample requires a much smaller volume, the “loop” is part of the valve structure and is not replaceable.

To change sample size, you must replace the entire valve.

Inlet Temperature

Gas samples

For gas samples, the inlet does not have to vaporize anything so the inlet does not have to be heated.

However, most chromatographers prefer to heat the inlet to ensure that nothing condenses in it. A temperature of 100°C is often used.

Liquid samples

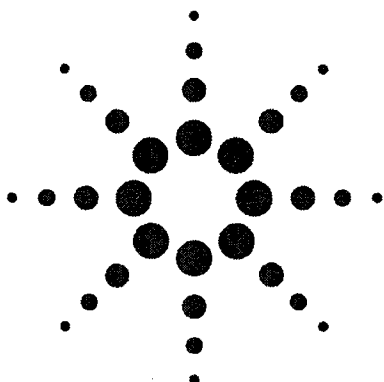
Liquid samples require a heated inlet. The temperature must be high enough to vaporize the sample but not so high that degradation occurs.

Hot enough Start with the solvent boiling point and examine the peaks. If they are all about the same shape (the sizes will differ), the inlet is probably hot enough. If the later peaks show excess broadening, raise the inlet temperature about 10°C to see if the shapes improve.

Too hot If you have more peaks than components and if they are poorly formed, suspect degradation problems.

Degradation in the inlet creates peaks whose size depends strongly on inlet temperature. To detect this, make a second analysis at a slightly lower temperature. Compare the peak sizes; any significant change could indicate degradation in the inlet.

2 Injecting Samples



3 Separating Components

| | |
|--|----|
| How a Column Separates Components | 28 |
| The basic principles of chromatography | 29 |
| Column Types | 30 |
| Capillary columns | 30 |
| Packed columns | 30 |
| Column tubing | 31 |
| Column Characteristics | 32 |
| Column efficiency | 32 |
| Gas control | 33 |
| Column resolution | 34 |
| Column selectivity | 34 |
| Capillary or Packed? | 35 |
| Column Temperature | 36 |
| Isothermal oven | 36 |
| Programmed oven | 37 |

The separation of a mixture into individual components occurs in the column. Many columns are available to separate mixtures. The choice depends on the nature of the mixture and the kind of information desired. However, all columns function using the same basic mechanism.



How a Column Separates Components

This is a cross-section of a column containing a two-component injected sample (the colored dots). There is no packing or coating; the column is just an empty tube (Figure 12).

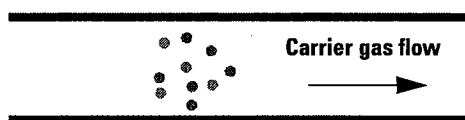


Figure 12 An uncoated column

If we look again a few seconds later, the appearance has changed (Figure 13).

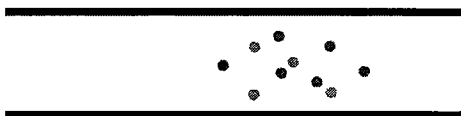


Figure 13 A few seconds later

The "sample" has moved to the right because of the carrier gas flow. It has broadened because of the concentration difference between the sample and the pure carrier gas surrounding it.

The components are still mixed.

Now we add a thin coating of a high-boiling substance on the inside surface of the column and repeat the experiment (Figure 14).

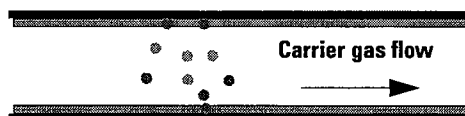


Figure 14 A coated column

We can use any coating we wish. In this case, we select one that dissolves the blue-dot component but not the yellow-dot component.

The blue-dot component distributes itself between the coating and the gas. The yellow-dot component stays in the gas phase.

When we examine the column a few seconds later, we find this: Figure 15.

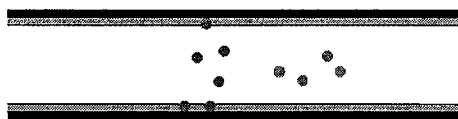


Figure 15 A few seconds later

The yellow-dot component is not attracted to the coating. It moves through the column at the speed of the carrier gas and will emerge first.

The blue-dot component divides its time between the stationary coating and the carrier gas. It travels through the column at a slower speed and will emerge later.

The sample has begun to separate into two peaks.

The basic principles of chromatography

- When a vaporized component is presented with a gas phase and a coating phase, it divides between the two phases according to its relative attraction to the two phases.
- The “attraction” can be solubility, volatility, polarity, specific chemical interaction, or any other property that differs from one component to another.
- If one phase is stationary (the coating) and the other is moving (the carrier gas), the component will travel at a speed less than that of the moving phase. How much less depends on the strength of the attraction.
- If different components have different “attractions”, they will separate in time.

Column Types

Capillary columns

A capillary column is an open tube with the stationary phase coated on its inside surface. There is no packing.

These columns range from about 0.1 to 0.5 mm inside diameter. A typical column length is 30 m. See Figure 16.

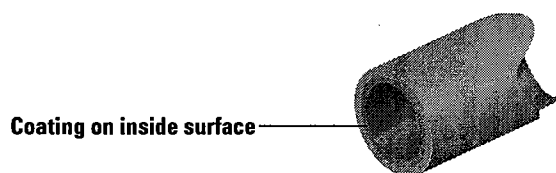


Figure 16 A capillary column

Capillary columns produce very narrow peaks. This allows the separation of very complex mixtures. For example, a typical automobile fuel yields between 400 and 500 peaks.

These columns, when made with fused silica tubing, are very inert. Difficult samples such as mercaptans, which tail severely on metal or glass columns, separate to the baseline on such columns.

Capillary columns require smaller samples than packed columns. A special inlet, see "Split/Splitless port" on page 19, allows a convenient-sized sample to be divided before it enters the column.

Packed columns

In a packed column, the stationary phase is coated on a finely-divided inert material to maximize its area and minimize its thickness. The coated material is then packed into a metal, glass, or plastic tube. See Figure 17.

Most metal packed columns are either 1/8- or 1/4-inch outside diameter. Glass columns are generally 1/4-inch outside diameter, but the inside diameter varies to produce the equivalent of the two metal column sizes.

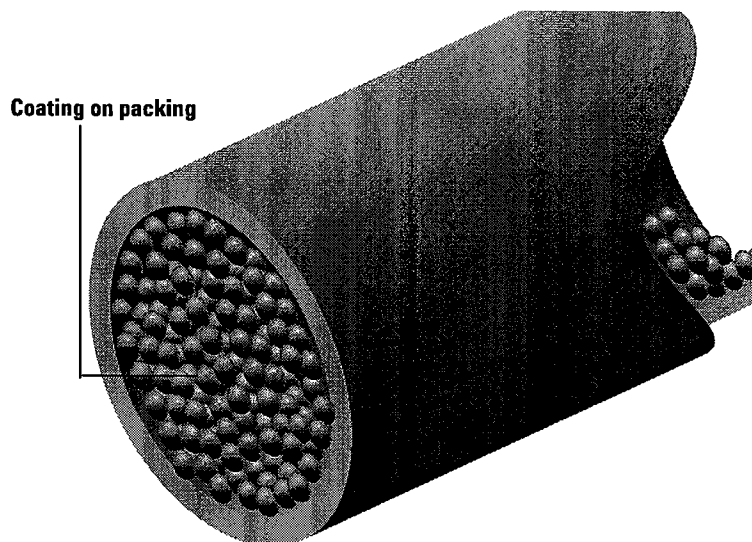


Figure 17 A packed column

Packed columns have high sample capacity, a necessity with older, less sensitive detectors. However, with modern high-sensitivity detectors, this advantage has vanished. Packed columns are still useful for gas samples, but capillary columns offer better resolution for most liquid samples.

Column tubing

Possible tubing materials include:

- Stainless steel—durable, but a relatively reactive surface may cause component loss or peak tailing.
- Glass—fragile, and usually requires treatment to deactivate the surface.
- Fused silica—used only in capillary columns, inert and robust, the preferred material for most uses.

Column Characteristics

The purpose of a column is to produce narrow, well-separated peaks from a multi-component sample. These two purposes are not entirely separate.

Column efficiency

A high-efficiency column produces narrow peaks. See Figure 18.

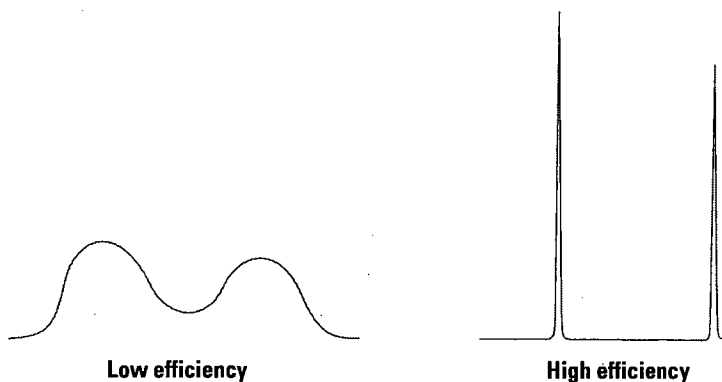


Figure 18 Column efficiency

Efficiency is determined by the column construction (small tubing diameter and thin stationary phase layer is best) and by the carrier gas flow rate.

See Table 2 for recommended flow rates.

Table 2 Recommended carrier flow rates

| Type | Diameter | Carrier flow rate, mL/min | | |
|-----------|-------------|---------------------------|------------|-------------|
| | | Hydrogen | Helium | Nitrogen |
| Packed | 1/8-inch od | 30 | 30 | 20 |
| Packed | 1/4-inch od | 60 | 60 | 50 |
| Capillary | 0.05 mm id | 0.2 to 0.5 | 0.1 to 0.3 | 0.02 to 0.1 |
| Capillary | 0.1mm id | 0.3 to 1.0 | 0.2 to 0.5 | 0.05 to 0.2 |
| Capillary | 0.2 mm id | 0.7 to 1.7 | 0.5 to 1.2 | 0.2 to 0.5 |
| Capillary | 0.25 mm id | 1.2 to 2.5 | 0.7 to 1.7 | 0.3 to 0.6 |
| Capillary | 0.32 mm id | 2 to 4 | 1.2 to 2.5 | 0.4 to 1.0 |
| Capillary | 0.53 mm id | 5 to 10 | 3 to 7 | 1.3 to 2.6 |

In each range, the lower value is close to optimum for the carrier gas and column combination. The higher value speeds the analysis without sacrificing very much efficiency.

Even higher flows, above the range given, can be used when separation is great or a shorter column is used. Flows below the range given increase analysis time and may cause an abrupt loss of efficiency.

Gas control

Flow in packed columns is usually controlled using mass flow controllers. Capillary columns, because of the very low flow rates, are usually pressure-controlled.

Some GCs provide electronic pneumatic control (EPC). Such instruments allow setting flows from a keyboard and reading them on a display.

Column resolution

A high-resolution column separates peaks down to the baseline. This is much easier if the peaks are narrow (the column is efficient).

A small change in flow rate can have an appreciable effect on resolution.

Combining the mathematical definitions of efficiency and resolution yields an important result:

Column resolution is proportional to the square root of column length.

This means that increasing column length is *not* an effective way to improve resolution. Doubling the column length doubles the analysis time (and the column cost) but only increases resolution by about 40%.

Column selectivity

This is a less clearly defined property of the stationary phase. Essentially, it is how well a phase differentiates between two compounds. Low selectivity—they elute together. High selectivity—the peaks separate.

Capillary or Packed?

Both have their places. Here are some of the considerations.

- Gas analyses are usually done using packed columns. They have the sample capacity to accommodate the rather large gas samples. Common packings for gas analysis include:
 - Molecular sieve—oxygen, nitrogen, helium, hydrogen, CO₂, CO, methane, etc.
 - Alumina—propane and up.
 - Porapaks—ethane, butane, CO₂, etc.

Some, but not all, of these can be used in capillary columns.

- Capillary columns have higher resolution than packed columns. Even with little selectivity, an adequate separation is often obtained.
- One capillary column can perform a variety of analyses that might require a collection of several packed columns to achieve.
- Useful stationary phases for both capillary and packed columns include:
 - Methyl silicones—non-polar to moderately polar
 - Phenyl methyl silicones (5 to 50% phenyl)—olefins, aromatics, to moderately polar
 - Carbowax (polyglycol)—acids, very polar
- The high resolution of capillary columns often permits trading resolution for time. Since resolution depends on the square root of length, an excellent capillary column can be cut into two very good capillary columns with only a minor loss in resolution. Analysis time is reduced to one-half!

Column Temperature

The stationary phase (coating) in the column has a preferred temperature range.

- The *minimum* temperature is usually a melting point. Below this, you are doing gas/solid chromatography; above it, you are performing gas/liquid chromatography. Results can be quite different.
- The *maximum* temperature is usually related to a boiling or degradation point.

Columns are mounted in a temperature-controlled oven because separations are highly temperature dependent.

The oven temperature can be isothermal or programmed. See Figure 19.

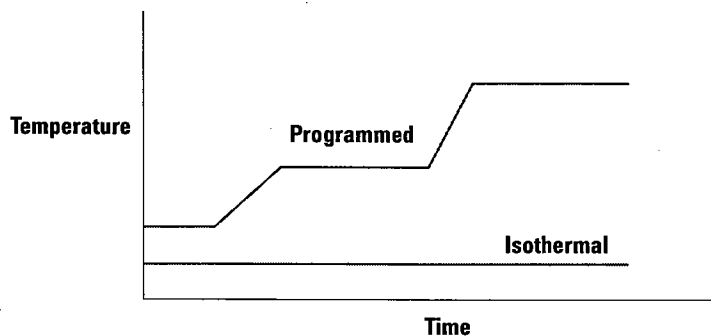


Figure 19 Oven temperature

Isothermal oven

This is the simplest way to run the oven. The oven remains at the same temperature throughout the analysis. It has *advantages*:

- The oven is always ready for a sample analysis.
- There is no recovery time between analyses.

And *disadvantages*:

- Samples with a wide range of component times take a long time to run.
- Because peaks broaden with time, later peaks may be difficult to detect or measure.

Programmed oven

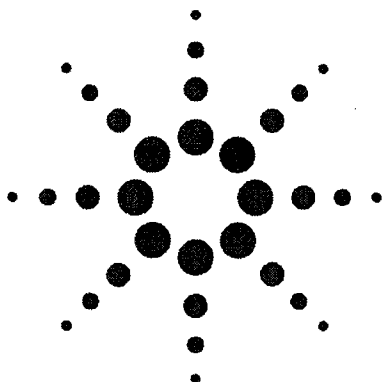
The oven temperature changes, usually upward, during the analysis. The *advantages* are:

- Analysis time is reduced.
- Peak shapes are constant throughout the run, making detection and measurement easier.

The *disadvantages* are:

- Components are subjected to higher temperatures than with an isothermal oven. This could cause degradation of sensitive components.
- The oven must cool to the starting temperature between runs. This cancels part of the time gained.

3 Separating Components



4 Detecting Components

| | |
|----------------------------|----|
| Thermal Conductivity (TCD) | 40 |
| How it works | 40 |
| Flame Ionization (FID) | 42 |
| How it works | 42 |
| Electron Capture (ECD) | 43 |
| How it works | 43 |

The three detectors described in this chapter do most of the GC work. They are augmented by others (see Table 3), mostly element-specific or mass-selective, which are not described in detail.

Table 3 Some other detectors

| Name | Uses |
|------------------------------------|---|
| Nitrogen-Phosphorus Detector (NPD) | Nitrogen- and phosphorus-containing compounds |
| Flame Photometric Detector (FPD) | Sulfur- and phosphorus-containing compounds |
| Atomic Emission Detector (AED) | Tunable for many elements |
| Mass Selective Detector (MSD) | Identify components from mass spectra; when combined with GC, the most powerful identification tool available |



Thermal Conductivity (TCD)

All gases conduct heat, but hydrogen and helium are, by far, the best thermal conductors (see Table 4). When either of these is used as the carrier gas, anything else that may be present causes a decrease in the thermal conductivity of the gas stream.

This change can be measured and used to create a chromatogram.

Table 4 Thermal conductivities of gases relative to hexane

| Gas | Relative thermal conductivity |
|----------------------|-------------------------------|
| Carbon tetrachloride | 0.44 |
| Benzene | 0.88 |
| Hexane | 1.00 |
| Argon | 1.04 |
| Methanol | 1.10 |
| Nitrogen | 1.50 |
| Helium | 8.32 |
| Hydrogen | 10.68 |

Since the TCD operates on thermal conductivity differences, it is clear that hydrogen or helium are the preferred carriers.

How it works

When a voltage is applied to a filament, it heats up. The steady-state temperature depends on the applied voltage, the resistance of the filament, and the rate at which the filament loses heat to its surroundings.

If a filament is immersed in a gas stream, any change in the thermal conductivity of the gas causes a change in filament temperature. This changes the resistance of the filament.

Early TCD designs used four filaments connected as a Wheatstone bridge. The column effluent flowed over two opposite filaments; pure carrier gas (the reference) flowed over the other two. When a component appeared, the bridge became unbalanced.

A modern TCD design is shown in Figure 20.

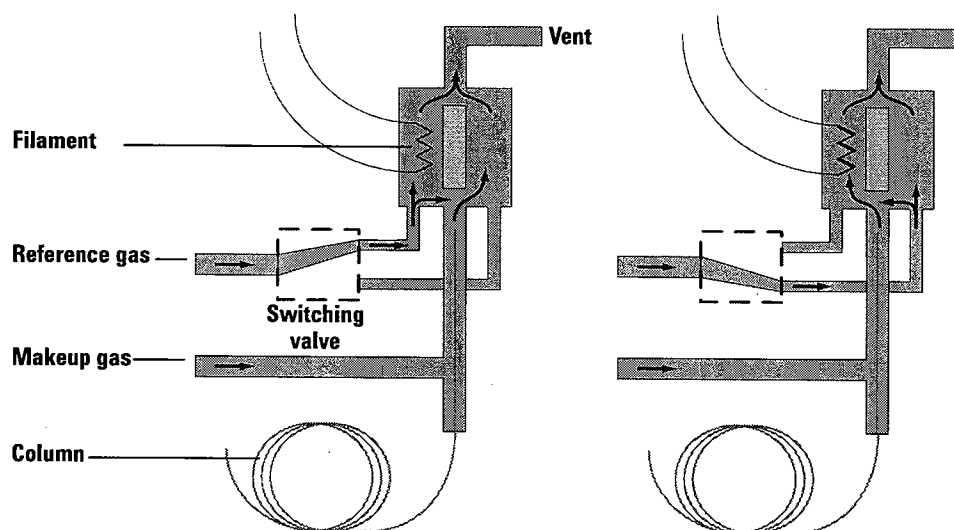


Figure 20 Thermal conductivity detector

This detector uses a single filament. A rapid switching valve causes it to sample the carrier effluent and a reference gas alternately. If the two gas streams are identical—no component present—the filament resistance does not change when the gases switch.

However, when a component enters the detector, the filament temperature drops when the column flow is switched in and then recovers when the reference gas is switched in. The electronics senses this change and adjusts the power to the filament to keep the temperature constant.

The power demand curve amplitude depends on the thermal conductivity difference between the column flow (when a component is present) and the reference gas.

Flame Ionization (FID)

An air/hydrogen flame creates very few ionized particles. However, if a carbon-containing material enters the flame, ion production increases.

How it works

The carrier gas from the column mixes with hydrogen and is burned in air. The FID uses two electrodes, one of which is often the jet where the flame burns, and a polarizing voltage to collect the ions from the flame.

When a component appears, the collected current rises. After amplification, the current creates the chromatogram.

The FID responds to anything that creates ions in a flame, which is essentially all organic compounds (there are a few exceptions).

A general FID design is shown in Figure 21.

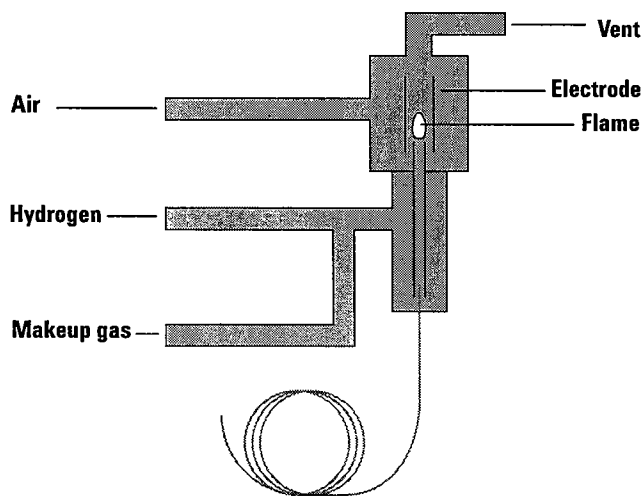


Figure 21 Flame ionization detector

Electron Capture (ECD)

The electron capture detector has found wide use in environmental work because of its very high sensitivity to halogen-containing components, which include most herbicides and pesticides.

How it works

A radioactive isotope, usually ^{63}Ni , in the detector cell emits beta particles. These collide with carrier gas to create showers of low-energy free electrons. Two electrodes and a polarizing voltage collect the electrons as a current.

Some molecules can capture low-energy electrons to form negative ions. When such a molecule enters the cell, some of the electrons are captured and the collected current decreases. After processing, this signal creates the chromatogram.

One form of the ECD is shown in Figure 22.

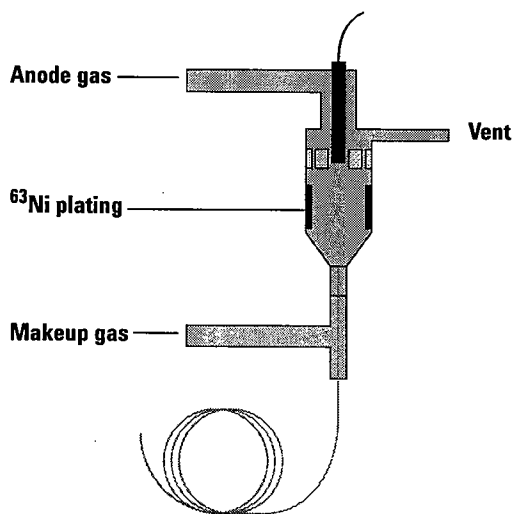


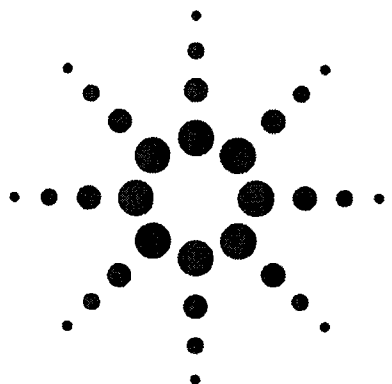
Figure 22 Electron capture detector

4 Detecting Components

The ECD is quite specific, responding strongly to anything that captures electrons and poorly to everything else. Some relative responses are listed in Table 5.

Table 5 ECD sensitivities to selected compounds

| Compound | Response relative to benzene = 1 |
|----------------------|----------------------------------|
| Benzene | 1 |
| Toluene | 3 |
| Acetone | 8 |
| 2,3-Butanedione | 800,000 |
| n-Butanol | 17 |
| Chlorobenzene | 1,200 |
| Bromobenzene | 7,600 |
| 1-Chlorobutane | 17 |
| 1-Bromobutane | 5,000 |
| 1-Iodobutane | 1,500,000 |
| Chloroform | 1,000,000 |
| Carbon tetrachloride | 6,600,000 |



5 Interpreting Chromatograms

| | |
|---------------------------|----|
| Peak Measurements | 47 |
| Retention time | 47 |
| Peak size | 47 |
| Component Identity | 49 |
| Component Amount | 50 |
| Uncalibrated calculations | 50 |
| Area and height percent | 50 |
| Calibrated calculations | 52 |
| Normalization | 54 |
| External standard | 55 |
| Internal standard | 56 |



5 Interpreting Chromatograms

The chromatograph produces a signal that varies with time. When plotted, it produces the familiar chromatogram (Figure 23).

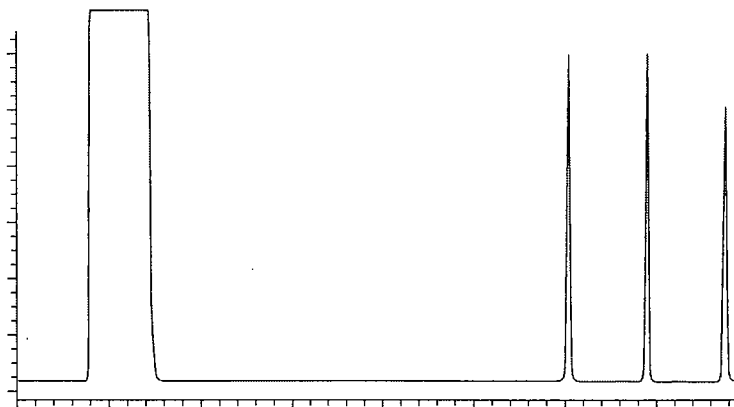


Figure 23 A typical chromatogram

The chromatogram can be converted into a list of peak times and sizes by either manual or electronic means.

Peak Measurements

Two basic measurements can be made on a peak:

- The time after injection when the peak is detected
- The size of the peak

Retention time

The *appearance time*, measured from injection to detection, is the sum of two parts:

- The *plumbing time*—how long it takes for the carrier gas to pass through the column. It is measured by injecting air or some other non-interacting substance.
- The *retention time*—the additional time caused by the component's interaction with the stationary phase in the column.

For most purposes, the plumbing time is ignored and the retention time is taken as the appearance time.

Peak size

Size can be measured either as peak area or peak height, both measured relative to a constructed baseline.

The baseline under the peak cannot be measured directly. It must be constructed from the baselines on either side of the peak.

This is simple with well-separated peaks. It is much more difficult when peaks are merged, on the trailing edge of a solvent peak, or otherwise less than ideal. For this reason, time spent improving the peak separation is time well spent.

Peak height

This is the simplest measurement, requiring only a ruler. It is the vertical distance from the top of the peak to the baseline.

Peak area

This is the area enclosed by the peak signal and the baseline under it. It is best measured by electronic means.

The two size measurements are shown in Figure 24.

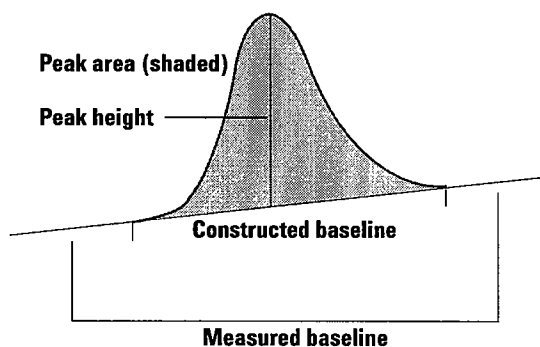


Figure 24 Measuring a peak

Integrators and data systems

Integrators excel at measuring peak areas or heights and peak retention times. They make the conversion of a curve (the chromatogram) into a table (of times and sizes) very simple and reproducible.

Data systems offer the same advantages and considerably more.

- A software integrator is more flexible than that in a hardware integrator.
- Data can be reprocessed using different integration and calculation parameters without re-injecting the sample.
- Systems perform the fundamental calculations described in this chapter, often with more sophisticated features.
- Systems produce user-designed formatted reports.
- Peak calibration becomes a very simple process.
- Both single- and multi-level calibrations are possible.
- Raw and processed data can be archived for later use.
- Systems process data from multiple GCs at the same time.

Component Identity

Because many compounds may elute at the same (or nearly same) retention time, gas chromatography by itself does not usually provide identification of a totally unknown sample.

However, it is a very powerful tool when the problem is more constrained. GC patterns can be compared to identify samples that have a high probability of being the same. For example, crude oil from a tanker can be compared to an oil slick on the ocean to determine if that tanker was responsible for the spill.

GC is quite useful for *eliminating* possibilities. If you know from previous experiment that iso-octane appears at 1.9 minutes, then the unknown peak at 1.5 minutes is definitely *not* iso-octane. But what is it?

Fortunately, you do not have to consider the entire universe of chemical compounds. Sample information limits the list of possibilities. For example, you would not expect to find streptomycin in a paint sample.

When a tentative peak identification has been made, it should be confirmed by repeating the analysis using a column that separates on a different basis. If a component has the right retention time on a boiling point column (methyl silicone) *and* on a polarity column (polyglycol), the identification is probably correct.

GC is especially useful in problems where the expected components are known and quantitation is required. GC will also usually detect the presence of unexpected components (as extra peaks).

Finally, GC can be connected to mass spectrometers or other selective detectors to provide additional data needed for positive identification of unknown components.

Component Amount

Uncalibrated calculations

A detector produces a signal while carrier gas is passing through it. If there is no component at the moment, the signal is the baseline. When a component appears, the signal increases.

The area between the projected baseline and the signal, while the component is passing through, is the peak area. The maximum vertical distance between the signal and the projected baseline is the peak height.

An integrator or data system handles the sometimes very difficult task of drawing the projected baseline, then measures the peak areas and heights. The results are the **Measured Responses (MR)**.

Area and height percent

Each peak is expressed as a percent of the total measured area or height in the run.

The detector is assumed to be equally sensitive to all components. Equation 1 shows the calculation.

$$\text{Amount of peak } n = \frac{[\text{MR of peak } n / \text{Sum of all MRs in the run}] \times 100}{(1)}$$

Advantages

- Fast setup, since no calibration is needed.
- Moderate sample size variation does not affect results.

Disadvantages

- All peaks must be detected.
- Any peaks not detected or not flushed from the column reduce the sum of MRs. This causes overestimation of all measured peaks.

Uncalibrated calculations do not correct for component sensitivity differences. This tends to overestimate the early peaks.

Common uses

- Generating a list of responses and retention times for building a calibration table.
- Analyses where the purpose is fast, reproducible results to be compared with preset limits.
- Useful in process monitoring, product release testing, etc.
- Not useful when absolute accuracy is important.

Calibrated calculations

If Area% and Height% are not adequate, the calibrated calculations use data from standard analyses to create individual peak calibrations.

The simplest calibration is the Response Factor, which is calculated by dividing the known amount of a component by the size of the peak it produces.

Graphically, it is the slope of a plot of component amount versus peak size, as shown in Figure 25.

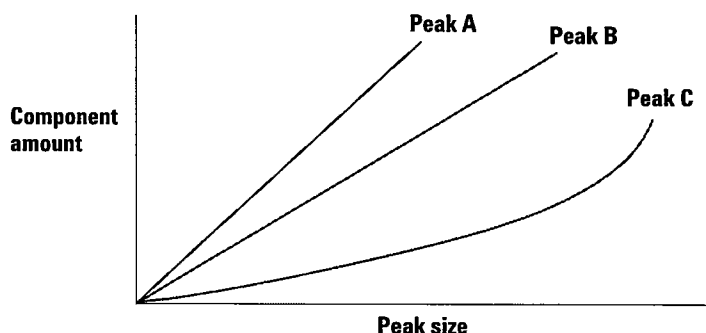


Figure 25 Response factors

Response Factors can be determined by analyzing a single standard mixture containing all of the components to be calibrated.

However, the Response Factor approach makes two important assumptions:

- The amount/size line passes through the origin.
- The amount/size line is straight.

For a trustworthy calibration, both assumptions must be demonstrated experimentally. If the line is really straight and really does pass through the origin, then the response factor is valid.

In Figure 25, Response Factors can be used for peaks A and B, but not for peak C. The two forms of calibration correction are shown in Equation 2 and Equation 3.

For Peaks A and B:

$$\text{CR of peak} = \text{MR of peak} \times \text{Response Factor of peak} \quad (2)$$

For Peak C:

$$\text{CR of peak} = \text{<Response Curve amount> of MR of peak} \quad (3)$$

Peak C can only be corrected by using the entire calibration curve. This is laborious by hand, but is easily done using a data system.

Normalization

The normalization percent is similar to Area% and Height%, but uses Corrected Responses instead of Measured Responses, as shown in Equation 4.

$$\text{Amount of peak } n = \frac{\text{CR of peak } n}{\text{Sum of all CRs in the run}} \times 100 \quad (4)$$

Advantages

- This calculation corrects for component sensitivity differences, which yields more accurate results for early peaks.
- Moderate sample size variation does not affect results.

Disadvantages

- The method must be calibrated.
- All peaks must be detected. Any peaks not detected or not flushed from the column will reduce the sum of CRs. This causes overestimation of all measured peaks.
- All peaks must be identified and calibrated to achieve the highest accuracy. Unknown (and therefore uncalibrated) peaks reduce the absolute accuracy of the calculation.

Common uses

- Provides very accurate results if there are no high-boilers to worry about.

External standard

The great advantage of external standard is that only the peaks of interest need to be calibrated. The calculation is very simple; see Equation 5.

$$\text{Amount of peak } n = \text{CR of peak } n \quad (5)$$

Advantages

- Only the peaks of interest must be calibrated.
- Only the peaks of interest must be eluted and measured.
- Each calibrated peak is computed independently.

Disadvantages

- Peaks of interest must be calibrated.
- The calculation assumes that instrumental drift is negligible. Known check samples must be run regularly to confirm this.
- Constant sample size is essential, since this is an absolute (rather than relative) calculation. This is very difficult to achieve using manual injection. In practice, a gas or liquid sampling valve or an automatic liquid sampler is a necessity.

Common uses

Gas analyses using a sampling valve. As instrument stability improves, and with the help of automatic injection devices to ensure constant sample size, ESTD is taking over many analyses that formerly required ISTD.

Internal standard

Internal standard provides independent calculation of each calibrated peak. It also corrects for variation in sample size, instrument drift, and other factors.

ISTD is considered the most accurate chromatographic calculation, although ESTD with modern equipment is rapidly improving.

The basic calculation is shown in Equation 6.

$$\begin{aligned} \text{Amount of peak n} = \\ \text{[CR of peak n / CR of ISTD peak] x Amount of ISTD} \\ \text{peak} \end{aligned} \quad (6)$$

The quantity **Amount of ISTD peak** is a known amount of the internal standard compound that is added to each sample before analysis.

This is generally considered to be the most accurate of the calculations.

Advantages

- Only the peaks of interest must be calibrated.
- Only the peaks of interest must be eluted and measured.
- Each calibrated peak is computed independently.
- Minor variation in sample injection size does not affect results.
- Minor instrumental drift does not affect results.

Disadvantages

- Peaks of interest must be calibrated.
- A known amount of an internal standard substance must be added to every sample.

Common uses

Liquid sample analysis where high accuracy is required.

Note

The term internal standard has come to have two slightly different meanings:

- 1 ISTD was originally developed to compensate for differences in manual sample injection size. To do this, the internal standard was added to the ready-to-inject sample after any sample workup (distilling, extracting, etc.) was completed. The main requirements for the internal standard were that it not be present in the original sample and that it produce a well-defined peak that is well resolved from the sample peaks. It did not have to be chemically similar to the sample components.
- 2 In many biochemical and related applications, the internal standard is added to the raw sample before sample workup. In this case, it must be chemically similar to the sample so that it will be affected by the workup steps in much the same way. Now the internal standard is being used to correct for two different things: variation in percent recovery during workup and sample size differences in the injection. This is not possible with a single standard. By precisely controlling the sample workup process and experimentally confirming that percent recovery is highly reproducible, that source of error can be reduced to an acceptable level.

5 Interpreting Chromatograms



Agilent Technologies

© Agilent Technologies, Inc.

Printed in USA, May 2002



G1176-90000

Commonly Practiced Quality Control and Quality Assurance Procedures for Gas Chromatography/Mass Spectrometry Analysis in Forensic Urine Drug-Testing Laboratories

B. A. Goldberger

Department of Pathology, Immunology, and Laboratory Medicine
University of Florida College of Medicine
Gainesville, Florida
United States of America

M. A. Huestis

Addiction Research Center
National Institute on Drug Abuse, National Institute of Health
U.S. Department of Health and Human Services
Baltimore, Maryland
United States of America

D. G. Wilkins

Department of Pharmacology and Toxicology
and
Center for Human Toxicology
University of Utah
Salt Lake City, Utah
United States of America

TABLE OF CONTENTS

| | | |
|------|---|----|
| | INTRODUCTION | 60 |
| I. | METHOD VALIDATION | 60 |
| | A. Assay Characterization | 60 |
| | B. Accuracy and Precision of the Analytical Method | 61 |
| | C. Linearity of the Analytical Method | 62 |
| | D. Specificity of the Analytical Method — Interference Studies | 63 |
| | E. Sensitivity of the Analytical Method — Limit of Detection and Limit of Quantitation | 63 |
| | F. Carryover | 65 |
| | G. Other Factors | 65 |
| II. | INSTRUMENT PERFORMANCE | 67 |
| | A. Instrument Checks | 67 |
| | B. Instrument Performance Evaluation and Tuning | 67 |
| | C. Chromatographic Performance | 68 |
| III. | ASSAY CALIBRATION | 69 |
| IV. | QUALITY CONTROL | 70 |
| | A. Required Quality Control Samples | 70 |
| | B. Verification of Quality Control Material | 71 |
| | C. Evaluation of Quality Control Results | 71 |
| V. | CRITERIA FOR DESIGNATING A POSITIVE TEST RESULT | 72 |
| | A. Chromatographic Criteria | 72 |
| | B. Ion Ratios | 72 |
| | C. Mass Spectral Match | 72 |
| | D. Quantitative Result | 72 |
| VI. | MISCELLANEOUS FACTORS | 73 |
| | A. Data Presentation | 73 |
| | B. Dilution Protocols | 73 |
| | C. Reinjection of Extracts | 73 |
| VII. | REVIEW AND EVALUATION OF GC/MS DATA | 73 |
| | REFERENCES | 76 |
| | ABOUT THE AUTHORS | 79 |

Commonly Practiced Quality Control and Quality Assurance Procedures for Gas Chromatography/Mass Spectrometry Analysis in Forensic Urine Drug-Testing Laboratories

REFERENCE: Goldberger BA, Huestis MA, Wilkins DG: Commonly practiced quality control and quality assurance procedures for gas chromatography/mass spectrometry analysis in forensic urine drug-testing laboratories; *Forensic Sci Rev* 9:59; 1997.

ABSTRACT: Forensic urine drug-testing laboratories operate in a prescribed scientific and administrative manner to ensure accurate test results. All specimens positive by an initial immunoassay test must be confirmed by gas chromatography/mass spectrometry (GC/MS). To provide adequate control and verification of these analytical processes, laboratories must implement appropriate policies and procedures to be used in routine practice. This review describes the following topics regarding GC/MS analyses: method validation, instrument performance, assay calibration, quality control, criteria for designating a positive test result, sample and batch acceptance criteria, and GC/MS data review.

KEYWORDS: Accuracy, calibration, carryover, gas chromatography/mass spectrometry, GC/MS, internal standard, laboratory certification, limit of determination, limit of quantitation, linearity, precision, quality assurance, quality control, sensitivity.

INTRODUCTION

Laboratories accredited by the National Laboratory Certification Program of the United States Substance Abuse and Mental Health Services Administration (SAMHSA, formerly the National Institute on Drug Abuse, NIDA), Department of Health and Human Services (HHS) and the College of American Pathologists (CAP) must perform urine drug testing in a prescribed scientific and administrative manner. Testing of specimens under the CAP and HHS Guidelines requires initial testing by an immunoassay, followed by confirmation of all positive initial test results by gas chromatography/mass spectrometry (GC/MS) [70,72].

To provide adequate control and verification of the analytical process, laboratories must implement appropriate policies and procedures regarding GC/MS analysis. This review is intended to discuss the following topics: method validation, instrument performance, assay calibration, quality control, criteria for designating a positive test result, specimen and batch acceptance criteria, and GC/MS data review. Although this review focuses upon those aspects of quality control and quality assurance pertinent to the regulated drug-testing laboratory, many of the components specified below are directly applicable to any laboratory performing drug testing in biological specimens. Indeed, many of the guidelines reviewed below have been adapted from recommended practices for pharmaceutical methods from the Food and Drug Administration (FDA) and the United States Pharmacopoeia (USP),

as well as recommendations resulting from a 1988 Ad Hoc Committee Report to the American Academy of Forensic Sciences (Toxicology Section) and a 1995 Mass Spectrometry and Good Laboratory Practices Workshop organized by the American Society for Mass Spectrometry [9,69,73,74,80].

I. METHOD VALIDATION

A. Assay Characterization

Method development is a process of documenting or proving that an analytical method is acceptable for its intended purpose. For analytical methods to be implemented in laboratory-based regulatory drug-testing programs, the laboratory must be able to demonstrate that the chosen analytical method has the ability to provide *accurate* and *reliable* data. These data can then be used to identify drug presence in a urine specimen according to pre-established administrative reporting limits (cutoff concentrations). Therefore, it is critical that the laboratory identify the key assay characteristics which it will validate prior to implementation of the method into routine use. Also, the laboratory must clearly define the evaluation criteria for each of the key assay characteristics it has selected as part of its validation. It has been suggested that at a minimum, the key assay characteristics to be established and evaluated should include: the accuracy, precision, linearity, specificity, sensitivity, carryover potential, and ruggedness of the analytical method [29]. Additional

characteristics to be evaluated may include: the stability of the analyte under various analytical and storage conditions, identification and concentration of the internal standard(s) for the method, validation of use of partial (diluted) sample volumes, and estimated recovery of the analyte from the matrix [18].

Specific evaluation criteria for method validation generally accepted by the scientific community for an analytical method can vary depending on the particular technique used (i.e., HPLC, GC/MS, LC/MS), as well as its particular application. Much of the available published information with respect to details of validation protocols, such as the number of batches to be evaluated, the number of replicates, and specific acceptability criteria, is based upon chemical analyses performed on autoanalyzers, or HPLC systems, rather than GC/MS systems [2,18,38,41,78]. Therefore, the application of quality control principles to GC/MS analysis of urine specimens has been based largely upon professional consensus, or "generally accepted laboratory practice" in the drug-testing community. This is in some contrast to a formally and experimentally developed, literature-based approach to implementation of quality control principles to a specific technology. Nevertheless, as individual GC/MS methods for drugs of abuse in urine matrices have been developed and published in the literature, quality control principles have been selected and applied in a variety of ways to assist in the validation of methods and increase confidence in the data that are obtained.

B. Accuracy and Precision of the Analytical Method

Two of the most important assay characteristics to be determined during method validation are accuracy and precision. Together, accuracy and precision determine the error of an analytical measurement. Accuracy and precision are frequently considered together because they are interdependent in assessing the acceptability of a method. The *accuracy* of a method, as used in biopharmaceutical or drug-testing analysis, refers to the closeness of the measured value to the true value for the sample. More specifically, it is a measure of the degree to which a mean obtained from a series of experimental observations agrees with the "true" or "accepted" value of the quantity to be measured. *Precision*, on the other hand, refers to the variability of measurements within a set. It is most often used to demonstrate scatter or dispersion between numeric values in a set of measurements that have been determined under the same analytic parameters.

The accuracy and/or precision of an assay can be determined by comparing test results utilizing laboratory-prepared standards and controls with those obtained with an established reference method and/or by analysis of

standard reference materials, such as those available from the National Institute of Standards and Technology (NIST) and CAP [3,17,22,23,66]. Secondary checks may involve reanalyses of performance test specimens and comparison of laboratory results with target means obtained via alternate methods already known to be accurate. (Reanalysis of performance test samples, however, may be prohibited unless the laboratory has obtained prior approval from the submitting agency.)

Accuracy is generally expressed as the percentage difference from the actual value (%DFA) as shown below:

$$\%DFA = [(\text{Mean} - \text{Spiked}) / \text{Spiked}] \times 100$$

An alternate way to determine accuracy is to determine whether the measured mean value is statistically different from the actual value using a *t*-test at 95% confidence [37]. The assessment of accuracy must be carried out on mean values which have been calculated from replicate measurements of reference materials containing known concentration of analyte. At a minimum, triplicate measurements are necessary to establish a single mean value and standard deviation (SD) for any single target concentration. During validation of the assay, it is generally accepted practice to assess accuracy at two to three different concentrations of analyte.

The specific concentrations used for the accuracy evaluation are selected to test accuracy across the range of the standard curve (calibration curve) of the assay. It has recently been recommended that accuracy be assessed using a minimum of 9 determinations over a minimum of three concentrations (e.g., 3 concentrations with 3 replicates each) [65,68]. Other authors have recommended a minimum of 36 determinations over a minimum of 6 concentrations (e.g., 6 concentrations with 6 replicates each) [78].

As stated earlier, the acceptability criteria for accuracy and precision for an assay should be preselected by the laboratory. Generally, accuracy acceptability ranges in forensic urine drug-testing laboratories do not exceed 20% (by convention) of the target concentration. Many laboratories routinely use lower ranges, such as 10%. It should be noted that the acceptable accuracy range selected for initial method validation may differ from that selected for routine use (batch acceptance criteria). For example, a laboratory may require that accuracy be within 10% of the known concentration during method validation, and then choose to increase the acceptable range to 20% for routine daily analysis to accommodate both random and systematic error [38].

Precision of an analytical method is usually assessed in two ways: analysis of multiple measurements during a single analytical run (within-run precision) and analysis

of single, or mean, measurements over many runs (between-run precision). Precision is expressed as the percentage relative standard deviation (%RSD), also referred to as the coefficient of variation (CV), as shown below:

$$\%RSD = [\text{Standard deviation} / \text{Mean}] \times 100$$

Within-run precision can be considered a measure of the precision of an analytical method under optimal conditions. The between-run precision, however, is likely to be a better representation of the precision one might observe during routine performance of the assay because these data are generally subjected to a greater number of sources of variability. The lower the calculated CV, the greater the precision of the assay. Precision of an assay at concentrations below, at, and above the assay cutoff concentration can be determined by repeated analyses of quality control samples on a within- and between-batch basis. One approach to assessing between-run precision of the method is to perform triplicate measurements on three separate concentrations of analyte, across three separate analytical batches. Subsequently, the laboratory evaluates the acceptability of the precision of the method using a criterion selected *a priori*. Generally, within-run and between-run coefficient of variation values of <15% are considered acceptable [2,18,29,33,78]. However, because greater variability is to be expected as analyte concentrations approach the limit of detection (LOD) of the analytical method, the laboratory might choose to increase the acceptability criterion to 20% at its lowest measured concentrations [37].

Finally, an additional technique for evaluation of between-run precision data is to apply a one-way analysis of variance (ANOVA) of the data to ensure that results do not significantly differ between analyses [37,42].

C. Linearity of the Analytical Method

The full range of linearity of a method should be established during initial assay characterization and periodically thereafter with specimens containing drug analytes over a wide range of concentrations. Further, the practical range of linearity, referred to here as the daily linear range, should be documented with every batch based upon data obtained with standards and/or controls [29,65]. Acceptance criteria for evaluating linearity data must include review of chromatographic appearance, retention time, and ion ratio or full-scan spectra matching criteria, for example. Although some analytical procedures may require nonlinear calibration, it is conventional for forensic urine drug-testing laboratories to utilize a linear model and univariate regression for GC/MS analysis. In this model, the independent variable is concentration (X) and

the dependent variable is response (Y), i.e., the value determined by the value of the independent variable. Recommendations for linearity studies are noted below; issues with respect to acceptability of daily assay calibration (range) are considered in a subsequent section of this paper.

In practice, linearity should be established via visual evaluation of a plot of signals (response) as a function of analyte concentration. If a linear relationship appears probable by inspection of such a plot, test results should be evaluated by an appropriate statistical method, such as the method of least squares regression [6,7,29]. Other statistical approaches must be clearly justified by the laboratory. Data from the regression line, such as the correlation coefficient (r), coefficient of determination (R²), slope, and residual sum of squares, can also provide useful mathematical estimates of the degree of linearity obtained with the analytical method. In addition, since it is not uncommon to expect an increase in variance as a function of concentration, it may be more appropriate to perform a weighted (rather than unweighted) regression analysis to improve accuracy at the lowest concentrations studied [8].

During initial method validation, the laboratory typically analyzes a series of standards (calibrators) that have been prepared at known concentrations of analyte. Data are plotted and analyzed as just described to determine the upper and lower boundaries of linearity. A frequently used criterion for determining the upper and lower boundary limits in the pharmaceutical industry is the point at which the slope of the line deviates from the overall slope by not more than 5% [20]. However, this recommendation is not originally based upon GC/MS analysis. An alternative is to "reverse calculate" the individual concentrations of each standard using the generated regression line and determine whether each is in compliance with the acceptance criteria established for evaluation of quality control samples (e.g., such as $\pm 20\%$ of the target value). Outliers may be identified as those concentrations of analyte at either extreme (high or low) which are outside the 20% criterion. Acceptable linearity, therefore, is demonstrated when the correlation coefficient exceeds a defined value, such as 0.990, and quantitative concentration of each point falls within $\pm 20\%$ of the target value. The discussion regarding evaluation criteria for linearity can be found in Section III.

The laboratory uses this information to establish the range, or concentration interval, which will routinely be used for analysis of samples. Validating the method over a wider range than that used in daily practice provides increased confidence that the routine standard concentrations are well removed from nonlinear response concentrations. If the laboratory elects to perform the linearity

assessment on more than one occasion, a statistical test of linearity can be performed for each standard curve separately using a weighted ANOVA [8,42].

D. Specificity of the Analytical Method — Interference Studies

Specificity refers to the ability of the analytical method to accurately measure an analyte response in the presence of all potential sample components. All methods should at a minimum be investigated for potential interference by endogenous matrix components, as well as common compounds that are structurally similar to the analyte of interest. A complete review of interference studies published regarding forensic urine drug-testing analytes is beyond the scope of this paper; however, some examples are provided below to illustrate general principles.

The potential interference of endogenous urine components with the assay is most frequently assessed by evaluation of urine specimens from several sources (donors) that are known to be drug-free for the analyte of interest. Assessment of interference from structurally related compounds can be determined by fortification of urine with high concentrations (e.g., 1 mg/mL) of potentially interfering analytes and cutoff concentrations of target analytes, or with concentrations of analytes that are expected under therapeutic conditions. For example, possible interference with the measurement of amphetamine and methamphetamine may occur due to the presence of sympathomimetic amines such as ephedrine, pseudoephedrine, phenylpropanolamine, and phentermine [32]. Further, interference with the measurement of morphine and codeine due to the presence of opiate metabolites and synthetic 6-keto-opioids such as dihydrocodeine, hydromorphone, hydrocodone, oxycodone, and oxycodone has also been described [25].

The determination of potential interferents that are *not* structurally related to the analyte of interest is more difficult to establish. However, the urine drug-testing laboratory may consider evaluating the potential interference of common over-the-counter products, as well as frequently encountered compounds which produce fragment ions also produced by the analyte of interest. In addition, the laboratory may refer to literature reports for interferences experienced by other investigators and assess their method with the potential interfering substances [34,59,67,79]. Although a laboratory cannot be expected to anticipate all potential interferents with its analytical method, it should make its best effort to characterize them whenever feasible.

The problem of interfering substances may be addressed by employing more selective extraction methods,

chromatographic separations, or detection methods. For example, to eliminate potential-false positive amphetamine/methamphetamine results due to the presence of other sympathomimetic amines, aliquots of specimens can be treated with a solution of 0.035 M sodium periodate at room temperature, then subjected to extraction. In the presence of periodate, α -hydroxyamines undergo oxidative cleavage removing the potential interferant [24]. Recent evidence indicates that periodate oxidation should be conducted at pH 7 or lower to prevent possible formation of low levels of amphetamine from extremely high levels of methamphetamine that may be present in the specimen [57]. In addition, lowering the injection port temperature of the gas chromatograph, coupled with other preventative measures, eliminates artifactual production of methamphetamine in the presence of high concentrations of ephedrine and/or pseudoephedrine [24,57,67]. It should be noted that some reported interferences may be method-specific and thus will need to be evaluated by the laboratory on an individual basis, as appropriate.

E. Sensitivity of the Analytical Method — Relationship to Limit of Detection and Quantitation

An analytical method is determined to be *sensitive* if small changes in concentration cause large changes in analytical response. It is directly related to, and frequently defined as, the slope of the standard curve [38]. However, this definition does not account for the variability of a measurement. The limit of detection (LOD) and limit of quantitation (LOQ) are terms which are used to express the ability of the assay to detect small concentrations of analyte, as well as attempting to account for variability of measurement.

The *limit of detection* of a method is the lowest analyte concentration that produces a response detectable above the noise level of the system. The *limit of quantitation* is the lowest concentration of analyte that can be accurately and precisely measured. The LOD and LOQ of a method are dependent upon several factors, such as the electron multiplier voltage, the volume of specimen analyzed, the detector threshold, the type and condition of the chromatographic column, the concentration of analyte, the amount and type of internal standard, the extraction efficiency, and the individual instrument [54]. A significant change or modification to any of these factors will require reassessment of the LOD and LOQ for the method.

There are several approaches for establishing the LOD and the LOQ of an analytical method [1,44,48,65]; at least two of these are routinely used in forensic urine drug-testing laboratories. Recent publications have defined issues of concern regarding LOD and LOQ determi-

nation methods, and have described and compared the two most commonly used methods [1,44].

The first approach to establishing the LOD and LOQ of an assay is based on the measurement of the magnitude of analytical background noise. It is performed by analyzing an appropriate number of blank (drug-free) samples and calculating the standard deviation of these responses. In practice, determining LOD using this approach involves the analysis of negative urine specimens (obtained from at least ten different donors) over time. In this paradigm, the LOD is calculated as the mean of the detected amount or signal intensity plus three standard deviations ($\bar{X} + 3 \text{ SD}$) [48], where 3 is a factor for a 99.9% level of confidence. Similarly, LOQ is calculated as the mean of the detected amount or signal intensity plus ten standard deviations ($\bar{X} + 10 \text{ SD}$). An obvious limitation of this approach is that while this may be an adequate measure of the theoretical LOD of a method, actual concentrations of analyte in biological samples measured at this calculated LOD would be indistinguishable from zero measurements by a large probability [38].

The second commonly employed approach for the determination of LOD and LOQ is based on a signal-to-noise comparison [65]. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. In this context, a minimum of a signal-to-noise ratio of 3:1 or greater is generally agreed to be acceptable for LOD assessment; a signal-to-noise ratio of 10:1 or greater is generally agreed to be acceptable for LOQ assessment. In practice, this approach involves analysis of a series of samples containing low concentrations of analyte.

For GC/MS analysis in urine drug-testing laboratories, SAMHSA defines the lowest analyte concentration that meets signal-to-noise, chromatographic, retention time, and ion ratio or full-scan matching criteria, as the LOD. The lowest concentration, that meets all of the above criteria and quantitates within $\pm 20\%$ of the target concentration and measures within a specified coefficient of variation, is designated as the LOQ.

The first two approaches described above are routinely utilized in laboratories performing regulated urine drug testing, although the second approach is preferred over the first approach since it is based upon measurement of an actual analyte response, rather than the absence of a response. A third approach to determination of LOD and LOQ values is based on the standard deviation of the analyte response and the slope of the standard curve. In this case, the LOD is expressed as 3.3 times the standard

deviation of the response divided by the slope (S) of the calibration curve ($3.3 \times \text{SD}/S$). The LOQ is expressed as 10 times the standard deviation of the response divided by the slope of the calibration curve ($10 \times \text{SD}/S$). The slope is estimated from the standard curve of the analyte, and the standard deviation is estimated by analysis of blank specimens, as described for the first approach. Alternatively, the residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation. It has been recommended that if the detection limit of an assay has been estimated by calculation or extrapolation, such as with the first or third approach described above, this estimate should be subsequently evaluated and validated by the independent analysis of a suitable number of samples known to be prepared near or at the detection limit [65].

The LOD is an important assay parameter due to the use of this value to evaluate retest results. The LOQ is important for defining the minimum accurate quantitative value of the assay. For example, dilutions that produce results less than cutoff but equal to or greater than the LOQ may be utilized with the appropriate dilution factor to calculate test results if the LOQ control included in the batch is found to be acceptable. Dilutions that produce results below the LOQ are not acceptable; specimens must be reassayed at a lower dilution. At least one publication [18] recommends that the assessment of the LOQ be obtained using LOQ samples that are prepared *independently* from that included in the standard curve because the LOQ standard that is included in the standard curve influences the regression equation (and thus is no longer an independent measure). Also, since the LOD and LOQ values are influenced by a variety of factors, such as the individual instrument or detector, it may be advisable for the laboratory to assess these parameters on each instrument used for a particular assay, although a general consensus on this issue has not yet been reached.

Interestingly, there are no requirements and few specific recommendations regarding the number of analyses or analytical runs to be evaluated for LOD and LOQ determination. Suggested numbers of replicates of any single blank or standard range from 10 to greater than 20 [19,55,56]. However, from a statistical standpoint it may be advisable to perform these replicate measurements of each blank or standard, in three separate batches, followed by *t*-tests or one-way ANOVA to determine if the calculated LOD and LOQ values differ significantly over time. A two-way ANOVA may be used to determine if a bias is present between instruments.

Finally, in an effort to reduce interlaboratory LOD and LOQ variation, and to introduce a sample that assesses minimum performance in each confirmation batch,

SAMHSA suggested that the LOQ achieved by the laboratory must be a value equal to or less than 40% of the assay cutoff concentration [71]. To ensure acceptable performance, it is desirable to challenge the limit of sensitivity in each batch by including a quality control sample at this concentration to monitor day-to-day instrument and assay variance.

F. Carryover

The term "carryover" is used to refer to the contamination of a sample by a sample analyzed immediately prior to it [35,63]. In the urine drug-testing laboratory, the term "carryover limit" is used to delineate the concentration of analyte in a sample above which contamination may reasonably be expected to occur. There is at least one common approach to performing such studies that involves the analysis of standards prepared at increasingly higher concentrations of analyte, preferably reflecting the highest concentrations which a laboratory typically encounters during routine analysis of samples. Each standard should be injected separately, followed by injection of a blank or solvent to determine if a signal (response) characteristic of the analyte is present in the sample above a pre-established limit (typically the LOD of the analytical protocol). Once the concentration at which carryover occurs is determined, the laboratory establishes its carryover limit at the next lowest concentration which *does not* have evidence of carryover in the blank or solvent. More precisely, upon completion of carryover studies, a laboratory should define the range of analyte concentrations at which carryover does not occur.

The laboratory should also ensure that the quantitative value for the carryover limit established in the carryover study falls within the linearity of the assay to ensure that the quantitative value is accurate. It is also advisable to evaluate carryover of an assay on each instrument system, including autosamplers, on which the method is to be performed, although there is no general consensus on this issue. This is to ensure that the established carryover limit is properly applied to data obtained on each system routinely used in the laboratory.

To minimize potential carryover, one or more of the following approaches can be utilized:

1. Use extensive solvent wash procedures between injections.
2. Inject solvent between all subject specimens.
3. Dilute the specimen prior to extraction.
4. Periodically determine or reassess the minimum carryover concentration.
5. Assay specimen extracts in ascending concentration order (according to initial immunoassay test results).
6. Reinject all highly concentrated specimens followed

by solvent blanks or negative quality control samples.

7. Assay a carryover standard followed by a solvent blank or negative quality control sample with each batch to assess carryover at the time of testing.
8. Frequently monitor the level of solvent available in the rinse vial for the autosampler to ensure that a sufficient quantity is available for the entire run.

It is important that criteria be established for evaluating the acceptability of solvent blanks or negative quality control samples that have been inserted to assess possible carryover [80]. If carryover is suspected, a potentially contaminated specimen should be re-extracted, rather than reinjected, because the extract vial may have already been contaminated.

G. Other Factors

Other factors, such as selection of a derivatizing reagent, selection of the internal standard(s) for the assay, selection of ions to monitor for selected-ion monitoring or full-scan analysis, stability of the analyte under various storage conditions, estimation of recovery of the analyte from the matrix, and evaluation of the ruggedness of the analytical protocol, should also be determined during the validation of an analytical method [10,18,29,37,38,78].

Selection of a *suitable derivative* is a critical component of assay development and method validation. There are at least three major reasons for using a derivatized compound. First, the analyte can often be made sufficiently volatile to allow its introduction to the mass spectrometer by gas chromatography, permitting optimal separation of the analyte from possible interfering substances. This, in turn, usually increases the specificity, precision, and sensitivity of the assay. Second, the stability of the analyte during storage, isolation, and thermal volatilization can be enhanced via formation of the derivatized product. Third, the increase in molecular mass resulting from derivatization may be beneficial, providing ions which, by virtue of their higher mass, are more specific for the analyte [28,43]. In cases where two or more derivatives are possible, each should be tested to assess its stability, chromatographic peak shape, and mass spectral properties in the biological matrix. The ideal derivatization procedure should be convenient and rapid to perform, form a consistent and stable product in high yield, require small volumes, be selective for the analyte of interest, be safe to handle, and should not form by-products that interfere with the analysis [4,5,39,40,52].

The selection of a *suitable internal standard* is highly linked to the appropriate selection of a derivative for the assay (if necessary), as well the particular ions to be monitored for analyte identification and quantitation. An

ideal internal standard behaves identically to the analyte throughout the extraction, chromatographic separation, and ionization processes. Stable isotope internal standards appear closest to meeting these criteria. The isotope label exerts only a slight effect on the physical properties, yet the higher mass of the isotope-labeled ions of the internal standard permits them to be readily distinguished from analyte ions by the mass spectrometer [16,26,50,58]. Deuterium-labeled analogs [^2H] are most frequently used as internal standards in urine drug-testing laboratories; other isotope-labeled analogs, such as ^{13}C or ^{15}N , are not commonly used.

Several factors are important to consider when selecting the deuterium-labeled internal standard to be used in an assay. The isotope should not undergo exchange under any of the conditions under which it will be used, such as the extraction, derivatization, or chromatographic separation procedures, as well as at the mass spectrometer's ion source [26]. In addition, the isotope must be stable under routine storage conditions, so that exchange of deuterium and hydrogen does not occur [50]. The isotopic variant selected should have a molecular weight three or more mass units greater than the unlabeled compound because the naturally occurring heavy isotope content of organic compounds in general produces ions of significant intensity at one or two mass units above each carbon-containing compound in the analyte's mass spectrum [26,45-47]. It is therefore critical that the isotopic variant is of high purity (>99%) to prevent interference with the analyte of interest during the analysis. Also, the labeling should be in such a manner that the isotopic atoms are located in proper molecular structure so that, after the fragmentation or ionization process, a sufficient number of high-mass ions that retain the label are present in significant intensities and will not interfere with the intensity measurement of the corresponding ions derived from the analyte [46].

The laboratory must carefully evaluate the concentration of internal standard used in its assay to ensure that there is no contribution to analyte signal itself. In effect, the substance ratio of internal standard material to analyte should be selected to give the least imprecision of quantitative analysis and to afford equal ion signal responses during mass spectrometric analysis of the analyte. Under certain conditions, improved sensitivity may be observed by the addition of a large excess of isotopically labeled analogue to reduce adsorptive losses ("carrier effect"). However, this approach is not generally preferred in urine drug-testing laboratories, where a specific administrative cutoff value must be applied. When excess deuterated internal standard is used, analytical precision usually suffers [43], which would not be desirable when a specific quantitative cutoff is needed. Therefore, it is recom-

mended that the laboratory eliminate or minimize adsorptive losses, rather than add excessive amount of internal standard.

The choice of *ion or ions to be monitored* for GC/MS assays has an important influence on analytical specificity. Generally, ions of high, even mass-to-charge ratios have fewer possible origins and are therefore more likely to be characteristic of a particular analyte. The laboratory initially performs a preliminary ion selection based on full-scan mass spectrometric analysis. Ions of high mass-to-charge ratios and good intensity are the first choice for use in routine assays. It is recommended that laboratories using selected-ion monitoring utilize at least three characteristic ions for the analyte of interest, and a minimum of two characteristic ions for the internal standard [72].

Laboratories using full-scan GC/MS must identify those ions with sufficient signal intensity and high-mass to use for qualitative identification of the compound ("matching criteria"). The laboratory should be able to demonstrate that the full-scan spectra it achieves, and plans to use on a routine basis, is stable and reliable over time. Further consideration of these requirements is discussed in Section V.

While most forensic urine drug-testing laboratories are using electron impact ionization (EI) for GC/MS assays, chemical ionization (CI) MS may also be utilized as the mode of detection to improve assay sensitivity and specificity. Chemical ionization MS typically produces an intense molecular ion and only a few fragment ions; therefore, one or two analyte ions may be monitored. A further discussion of these two methods of ionization are beyond the scope of this paper; however, the use of CI is acceptable only if the selectivity, accuracy, and precision of the CI process and method have been fully evaluated [26,81].

Another important parameter to assess during the method validation phase is that of *stability of the analyte*. This includes stability of stock solutions of analyte as well as stability of the analyte in biological matrix. Stability studies will typically be performed to assess stability under different temperatures (storage conditions) and different lengths of time (in-process stability and long-term stability). Stability of the analyte can be assessed at room temperature, refrigerated, and frozen storage conditions. The length of time under each storage condition to be evaluated can range from days to weeks. It is recommended that the laboratory at least establish analyte stability under its own anticipated storage and processing conditions. Although there are many different approaches to the performance of stability studies, a common approach is to use quality control materials prepared at known concentrations to assess stability [18].

Recovery of the analyte from a biological matrix must be determined to ensure that it is adequate and consistent. It is recommended that recovery studies be performed across the range of the standard curve, preferably at the lowest, mid-range, and highest concentrations encompassed by the curve [18,61,78]. Typically, a set of samples of known concentrations is prepared in triplicate at three different concentrations, internal standard is added, and the sample then extracted, derivatized, and analyzed by the GC/MS procedure. A second set of samples is also concurrently analyzed; however, the internal standard is not added until just prior to derivatization. Recovery is then calculated by comparing the calculated concentrations of the two sets of samples and expressing total recovery of the method as a percent. Intermediate points in the extraction process may also be evaluated.

The *ruggedness and reliability of the assay* should be established. Critical assay steps need to be identified, including assessing the importance of pH, solvent mixtures, derivatizing reagents, and temperature and incubation times utilized during the hydrolysis and derivatization processes. In addition, the level of expertise required to perform the analysis needs to be assessed. It must be determined whether the assay can be stopped and restarted, and how long the derivatized analytes are stable. A system for monitoring assay performance variables such as the number of rejected batches, calibration curve parameters (including slope, y-intercept, and correlation coefficient), and quality control results must be established [42,65,78].

II. INSTRUMENT PERFORMANCE

A. Instrument Checks

In addition to developing validated assays for the purpose of identifying and quantitating drugs in urine, it is an essential quality assurance component that the laboratory monitor and document that all analytical instruments involved in the analysis are maintained and operated properly. The laboratory must establish that the instrument used for a particular analysis is operating adequately and within expected performance specifications. Prior to the start of an assay, the condition of the GC/MS system must be evaluated. The GC/MS operator should check the injection port, detector and oven temperatures, and carrier gas pressure, and perform routine maintenance, as needed, such as clipping of the GC column and replacement of the injector septum and liner. In addition, on a periodic basis, or when a new column is installed, the carrier gas flow rate should also be measured.

Routine maintenance should be performed at least as often as recommended by the manufacturer. Additional maintenance and instrument check schedules should be developed by the laboratory according to its workload and type of assay performed. For example, the laboratory may choose to routinely replenish or replace vacuum pump oil on a quarterly basis. Or, the laboratory may choose to replace injection port liners daily. Other types of maintenance procedures include replacement of filaments, cleaning of the ion source, and replacement of electron multiplier or ion gauge. The specific schedule developed by the laboratory will be dependent upon the type of GC/MS instrument used (e.g., traditional quadrupole, ion-trap), as well as the nature of the extracts analyzed on a particular system. The GC/MS laboratory must maintain records of all routine and nonroutine maintenance performed prior to analysis of specimens, as well as written standard operating procedures for the performance of these tasks [9,26,31,53,80]. Record keeping for maintenance procedures is vitally important to demonstrate the validity of the analysis [31].

B. Instrument Performance Evaluation and Tuning

On a daily basis, it is a good practice to check the pressure in the ion source and in the analyzer, as well as the GC column head pressure to assure that no major system leaks have occurred. For quadrupole operation, a pressure of 10^{-4} torr or better is required so that significant interaction between the ion beam and residual gas molecules does not occur (which causes scattering of the ion beam and loss of sensitivity). For GC/MS, an even lower pressure of 10^{-5} torr is necessary to reduce residual gas in order to prevent significant distortion of mass spectra and reduce background interference. Chemical ionization MS typically requires higher source pressure than EI MS.

Following this initial pressure check, the laboratory should verify that there are no significant air/water leaks in the system by monitoring the intensity of the following ions: m/z 28 (N_2^+), 32 (O_2^+), 40 (Ar^+), and 44 (CO_2^+). If these peaks are abnormally large, an air leak may be indicated. In addition, evaluation of water vapor in the system can be checked by monitoring m/z 18 (H_2O^+) and 19 (H_3O^+). Appropriate instrument maintenance must be conducted before analysis is permitted to proceed.

A GC/MS "tuning procedure" ensures that appropriate mass-to-charge assignments and abundances of specific ions have been established, as well as indicating the need for instrument preventative maintenance. In order to verify proper calibration and operation of the mass spectrometer, the instrument must be tuned daily with an appropriate tuning compound (e.g., perfluorotributyl-

amine, PFTBA) for proper unit resolution and mass assignment. "Resolution" refers to baseline separation between consecutive integral mass peaks. In mass spectrometry, resolution and sensitivity are inversely related. Tuning should be performed at the operating temperature of the ion source.

Autotune procedures typically utilize a preselected set of criteria across a range of mass-to-charge ratios (e.g., m/z 69 to 502 for PFTBA) to optimize source and quadrupole potentials. Alternatively, manual tunes can be used to increase sensitivity over a narrower mass range. Autotune procedures, or optimization of tuning parameters across a wide m/z range using a manual tune, are most useful for analysis of unknown specimens. Narrow-range manual tunes can be useful for some low-level target compound analyses. In this case, either of the abundant ions at m/z 219 or 414 can be used to optimize source potentials, depending on which is closest to the analyte and internal standard ions that will be monitored. Manual tuning is acceptable as long as the MS operator is appropriately trained, the tune procedure is fully documented, and the laboratory's standard operating procedure (SOP) manual describes the task clearly and accurately. It is most common, however, for urine drug-testing laboratories to perform an autotune across the entire mass range (e.g., m/z 69 to 502) at approximately 70 eV [9,26,31,43].

All tune reports should be reviewed thoroughly by the operator before testing is initiated to support compliance with manufacturer and laboratory specifications. Acceptable limits should be established for the ion focus, ion appearance and peak width, abundance of selected ions, isotope ratios, and mass slope, as appropriate for the GC/MS instrument. Critical tune values should be monitored on a regular basis, and all tune reports, including unacceptable ones, should be archived as important supporting forensic documents. Autotune, or manual tune, information should be available during review of the batch to ensure that instruments were performing as expected prior to analysis of specimens. These records may be filed with the pertinent batch, or filed in a manner to permit easy retrieval [72].

C. Chromatographic Performance

The chromatographic performance of an assay should also be assessed before the analysis of specimens. This is achieved readily by the injection of an unextracted performance standard including analyte(s) and internal standard(s). Use of an unextracted standard removes sources of variation due to the extraction procedure and matrix interferences. In addition to permitting evaluation of the quality of the chromatographic system, the analysis

of an unextracted standard serves to verify that the analyte(s) of interest elute at the expected retention time, that all MS acquisition windows are appropriately set, and that no unexpected adsorptive system losses have occurred. Evaluation criteria in the SOP manual should include a thorough description of peak shape, resolution, and signal abundance requirements.

One approach to evaluation of acceptable chromatography for a single specimen and/or an entire batch of specimens may be defined and assessed by the following criteria:

1. The analyte of interest is present at the correct retention time.
2. The peak of interest obtained from the total ion chromatogram and each individual ion chromatogram is inspected visually for geometric symmetry. A gaussian peak shape (see Figure 1) is required and shall have no greater than 50% tailing using the following procedure:
 - a. Draw a vertical line from the apex through the center of the peak to the baseline.
 - b. Draw a line parallel to the baseline at 10% of the peak height.
 - c. Measure the distance "a" from the leading edge to the centerline.
 - d. Measure the distance "b" from the centerline to the trailing edge.
 - e. Calculate the ratio of distance "a" and distance "b".
 - f. If the ratio is greater than 2 or less than 0.5, performance is unacceptable.
3. The peak of interest is inspected visually for the presence of unresolved peaks. The maximum allowable valley between two adjacent peaks must not exceed 10% of the analyte peak height.
4. Abundance or signal-to-noise levels of the internal standard ions must meet established minimum criteria.

Failure to meet any of these criteria may provide cause for reinjection or reextraction of a specimen. A complete review of methods for evaluation of chromatographic performance is beyond the scope of this paper; however, acceptable alternative criteria for evaluation of chromatographic acceptability can be found in many textbooks and other reference materials [30,36,49,51,60, 63]. Some instrument software designers are also developing programs for automated evaluation of chromatography.

In addition to daily chromatographic checks prior to batch analysis, performance of the chromatographic column should be evaluated upon installation and periodically thereafter. This can be accomplished by the analysis of a variety of "test mixtures," such as a Grob's Test Mix

[30], to assess column performance. These mixtures generally contain several compounds (e.g., early and late eluters) which are selected to evaluate certain aspects of chromatographic performance, including peak shape and sensitivity. From a practical perspective, the forensic urine drug-testing laboratory may also choose to evaluate acceptable chromatographic performance for selected drug analytes (e.g., amphetamine, phencyclidine, 6-acetylmorphine, codeine) after column installation. Deterioration in performance can then be followed by comparison of the initial chromatogram with subsequent chromatographic data.

III. ASSAY CALIBRATION

The term "*assay calibration*" refers to the process of developing a mathematical model that attempts to predict the value of an independent variable (e.g., concentration) based on the value of the dependent variable (e.g., peak height, peak area ratios). Calibration has sometimes been referred to as inverse prediction or discrimination. This is in contrast to the term "regression," which was described earlier in this paper with respect to linearity of the analytical method. *Regression* refers to a functional relationship between two or more correlated variables [6,21]. In forensic urine drug-testing laboratories, assay calibration is necessary to determine whether an analyte is present in an unknown sample at or above a pre-established administrative cutoff value, as well as to determine the accurate quantitative concentration of the analyte under certain circumstances [72]. Currently, it is most common to calibrate the assay for quantitative measurement using a single, abundant ion fragment that is characteristic of the analyte of interest. Additional ions, referred to as qualifying ions, are used to support the qualitative identification of the analyte in the unknown sample.

Three approaches to assay calibration are commonly utilized in forensic urine drug-testing laboratories: multi-point calibration curves, single-point calibration curves,

and historical calibration curves. The first and probably the most widely employed method of calibration is the preparation of a *multi-point calibration curve*. Experimentally derived multi-point calibration curves are used to cover the concentration range of the samples to be measured, thereby improving the confidence limits associated with the calibration itself. Calibration samples (standards) are prepared from mixtures of different amounts of known concentration of analyte with a fixed amount of internal standard. Specific ion-abundances are measured for the analyte and internal standard, a ratio calculated, and a calibration curve generated using a simple least squares regression model, for example. If the laboratory elects to use a multi-point calibration curve, it must include calibrators that bracket the cutoff concentration. Although many laboratories include a calibrator at the cutoff concentration, it is not required [72].

The calculated concentration of all standards utilized to construct the calibration curve should be within $\pm 20\%$ of their respective target concentrations. If more than three standards are utilized to construct a calibration curve, it is permissible to delete one calibrator for cause (e.g., poor recovery) provided it is not a calibrator at the cutoff concentration. It is not acceptable to eliminate a calibration point solely in order to bring the quality control results within range, although there is some disagreement on this issue since one might expect that the probability of detecting/obtaining an "outlier" will increase as the number of calibrators analyzed in a batch increases [15,72].

Evaluation criteria for linearity of the multi-point calibration curve include the correlation coefficient (r), coefficient of determination (R^2), the slope (m), y-intercept (b), and quantitative value of each data point. The *correlation coefficient* is a measure of the intensity of association between two variables, X and Y . In calibration, the correlation coefficient ranges from 0 to 1; 0 indicates no correlation and 1 indicates a perfect correlation. A correlation coefficient may indicate a high degree of relation between variables, but the generated model (regression equation) may give an inadequate fit for the data. In contrast, the *coefficient of determination* is a measure of "goodness of fit" and is the proportion of variation in the data explained by the regression model. It also ranges from 0 to 1, with 0 indicating a complete lack of fit and 1 indicating a perfect fit. Generally accepted criteria for acceptable linearity using deuterated internal standards for calibration include a correlation coefficient and/or coefficient of determination of at least 0.990, and a y-intercept close to zero, although slightly positive or negative y-intercept values are also acceptable. Because of the potential for negative and positive bias, y-intercept

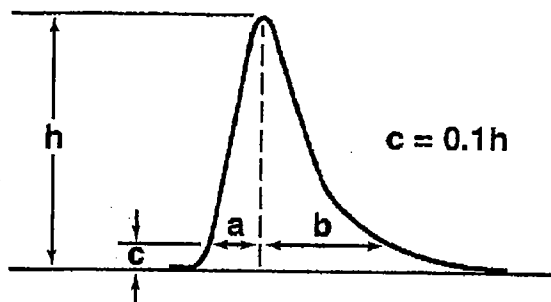


Figure 1. Theoretical chromatographic peak.

values should be monitored on a regular basis and tracked for development of trends.

For assay calibration, the laboratory must also establish whether the regression line will be forced through the origin (a "no-intercept" model) or will not be forced through the origin (an "intercept" model). In the intercept model, R^2 is the proportion of variance explained by the model. In contrast, R^2 produced by the no-intercept model is a measure of the degree of dispersion around zero and describes the proportion of variance around zero explained by the model [7]. Most GC/MS software programs allow for the user to select between these two approaches. The selection of the appropriate model to be used for a particular assay will depend on several factors, including the type and sensitivity of instrument, the analytical method, the method of ionization, the required analyte detection, and reporting limits. As a general rule, it has been suggested that one should assume that an intercept model is correct until proven otherwise via statistical testing of the intercept [6-8,14]. If statistical testing of the intercept indicates that it is not different from zero (e.g., $b = 0$), then the no-intercept model is appropriate and should be used. However, selection of a no-intercept model implies that the assay limit of detection is zero, which is not the case for GC/MS urine drug-testing assays due to instrument and matrix background effects. Therefore, in most instances regression through the origin should not be used in assay calibration.

The second most commonly employed approach to assay calibration is a *single-point calibration*. In this case, a single standard containing analyte at the assay cutoff concentration is used to establish the cutoff concentration in order to determine whether a specimen is positive or negative. Laboratories then include quality control materials at concentrations below, at, and above the cutoff concentration to demonstrate linearity. The quantitative results for the quality control samples used in single-point calibration must fall within $\pm 20\%$ of their respective target concentrations.

The third approach to assay calibration uses construction of a *historical multi-point calibration curve*. The laboratory establishes the calibration (as above) and verifies that the calibration has not changed between batches via analysis of control samples, one of which is at the cutoff concentration. Use of historical calibration curves is acceptable only if the laboratory has demonstrated linearity and precision of the calibration curve over time. If shifts in response ratios are observed or new internal standard materials are employed, a new standard curve must be prepared. In addition, all batches must include at a minimum a blank and two controls, one at or near the cutoff.

During assay calibration, the laboratory also establishes acceptance ranges for retention time and ion ratios. These acceptance ranges are then used to evaluate each calibrator, control, and unknown specimen in the batch (see Section V). For single-point calibration assays, the acceptable limits are determined from the calibrator at the cutoff. For multi-point or historical calibration curves, acceptable limits may be determined from either the calibrator at the cutoff or from the average of all the calibrators analyzed. If historical calibration curves are used, verification of correct retention time and ion ratios is performed by examination of the "at or near cutoff" quality control sample. In this case, the retention time and ion ratios of this sample must fall within the ranges established by the historical curve, or the assay must be recalibrated [72]. Any of the approaches for determining retention time and ion ratio criteria discussed above are considered acceptable; however, the laboratory must apply the acceptable ranges consistently to all calibrators, controls, and specimens in the batch.

IV. QUALITY CONTROL

A. Required Quality Control Samples

Each assay batch must include a minimum total of 10% open and blind positive and negative quality control samples in an appropriate urine matrix [70]. External blind quality control samples are not required, but are highly recommended. Quality control samples may be purchased commercially or prepared from a different source or lot of standard material other than that used to prepare calibrators. The use of different sources and lot numbers is recommended in order to eliminate systematic quantitative bias in the GC/MS assay that may otherwise go undetected. However, at a minimum, quality control materials should be prepared using standard material prepared from a different weighing or vial of source material other than that used to prepare the calibrators. The target concentration of at least one control must be within approximately 125% of the cutoff concentration; other controls should be prepared at appropriate concentrations in order to regularly assess accuracy below and/or above the assay cutoff concentration. It is recommended that a highly concentrated control sample be diluted in a similar manner as diluted specimens during aliquoting in order to verify the accuracy of the dilution technique, if one is routinely used to prepare presumptive positive specimens for confirmation [72].

In order to evaluate the efficiency of the hydrolysis process, cannabinoid and opiate control samples containing conjugated 11-nor- Δ^9 -tetrahydrocannabinol-9-car-

boxylic acid and morphine, respectively, should be assayed. Negative urine samples spiked with these reference materials can be prepared, or as an alternative, hydrolysis control samples can be prepared from a combined urine pool of previously confirmed specimens. (It is not acceptable to use regulated specimens for this or any other purpose until after completion of the required storage time.)

Finally, it is highly recommended, but not required, that the laboratory use quality control materials that include the addition of potentially interfering substances in its GC/MS assays. These might include compounds that are structurally related to the analyte of interest, or closely eluting compounds. For example, the laboratory may choose to include a control containing hydrocodone, hydromorphone, or oxycodone in its opiate assay to demonstrate that codeine and morphine are correctly identified and accurately quantitated in the presence of potential interferents. Another example is that of the inclusion of a control containing phenylpropanolamine, ephedrine, phentermine, and/or pseudoephedrine in GC/MS assays that utilize a periodate procedure.

B. Verification of Quality Control Materials

Prior to the use of reference materials to prepare calibrator, control, or internal standard material in the laboratory, the laboratory is required to verify, *independently* from the supplier, that its chemical identity is correct and that it is of acceptable purity and concentration. The laboratory may perform this verification itself, or may refer it to another laboratory. At a minimum, most laboratories perform a full-scan GC/MS analysis to verify the chemical identity and purity of the material and compare the obtained spectra with that of available library spectra to determine that significant impurities are not present in the material which might interfere with the method. The appropriate derivatization procedure for the analyte of interest is employed, as well as the GC conditions routinely used for the assay.

The isotopic purity of the internal standard can also be verified with the same procedure described above. In addition to full-scan analysis, the laboratory may also evaluate the deuterated internal standard in selected-ion monitoring (SIM) mode prior to use.

Additional methods for verification of chemical identity and purity may involve measurement of physical constants, such as melting point or refractive index, as well as use of other analytical techniques (HPLC, IR, NMR, TLC, or UV/VIS) to detect nonpolar or nonvolatile impurities [63]. Verification of concentration is most often evaluated indirectly by preparation of calibrators or

controls at known concentrations and analysis in routine batches.

The laboratory must establish specific evaluation criteria for reference materials, such as spectral match requirements, percent isotopic purity required, and quantitative results. The laboratory must retain documentation of all verification procedures performed [72]. The laboratory may then use the reference material to prepare calibrators for controls for routine use. Of course, these new calibrators and controls must then be themselves validated for concentration (e.g., $\pm 20\%$ of the target concentration) prior to routine use.

C. Evaluation of Quality Control Results

There are two major approaches to evaluation of quality control results applied to urine drug testing. The first approach is the use of a fixed-criterion quantitative acceptance range. In this case, the measured concentration of control samples must be within $\pm 20\%$ of the target concentration. A more detailed discussion of the rationale underlying this criterion has already been discussed in Section I-B.

The second approach is to use modifications of Westgard Quality Control Rules to evaluate results. In this approach, the laboratory establishes warning limits and out-of-control limits for the assay based upon the validated mean and standard deviation for the control sample. A thorough description of Westgard rules may be found in several sources [15,35,36,75-77]. Westgard rules are usually not directly applicable to GC/MS forensic testing due to the limited number of quality control data points obtained in a batch, the large number of independent variables associated with GC/MS systems, and the acceptance of potentially out-of-range data. However, if *coupled* with a fixed quantitative accuracy requirement of $\pm 20\%$ for control materials, the rules can be extremely useful for the purposes of evaluating the GC/MS assay for development of trends and systematic biases [38]. Also, other approaches to the evaluation of quality control data, including an ANOVA approach, have been described [37,42].

The laboratory's SOP manual must thoroughly describe the quality control evaluation criteria to be used and must include a policy for the required course of corrective action if quality control sample results fail to meet acceptance criteria. In order to assess laboratory performance, all control data, including out-of-limit data, should be recorded in the quality control log in Levey-Jennings or Shewart chart format [15,27,62,72,75-77]. Out-of-limit data should include documentation of required corrective action. It may be acceptable to reinject a quality control

sample one additional time. If the results are still unacceptable, other minimally acceptable protocols include:

1. Reinjection of calibrators, followed by reprocessing of *all* quality control samples and routine specimen data against the new calibration, *if* the time since the last injection is not excessive and the instrument has not been retuned;
2. Reinjection of all calibrators, quality control samples, and routine specimens; and
3. Acceptance of negative test results that are less than the LOD and reextraction of all other specimens in the batch.

V. CRITERIA FOR DESIGNATING A POSITIVE TEST RESULT

A. Chromatographic Criteria

Criteria for designating a positive test result include chromatographic and spectral identification. Chromatographic identification of an analyte requires comparison of the retention time of the specimen with that of a calibrator at the cutoff or the average of multiple calibrators (either approach is considered acceptable). Generally, the retention time of the analyte should be within $\pm 2\%$ of the retention time as established by the calibrator(s) [11-13,72].

B. Ion Ratios

Further, identification of an analyte requires comparison of ion ratios or full spectral data of the unknown with preestablished ion ratios or full-scan mass spectra, respectively. Acceptable ion ratios for the analyte and its corresponding internal standard are usually calculated using ion abundance data obtained for the standard prepared at the cutoff concentration or by determining the mean ion ratios for all calibrators. It has been demonstrated statistically that while full-scan mass spectrometric data provide the maximum confidence for analyte identification, a minimum of three structurally significant ions generated under electron ionization conditions appear to provide adequate information for an identification [12,64]. If the regulated urine drug-testing laboratory uses EI, it is currently required to use a minimum of two ion ratios for identification of the analyte and at least one ion ratio for the internal standard [72]. Of course, CI-generated spectra do not always meet the three-ion criteria because less extensive fragmentation is generally observed with this technique. However, this limitation is offset by the production of high m/z ions characteristic of the analyte and increased specificity with selection of appropriate reagent gases [11,12,26,81].

The ion ratios for the analyte and its corresponding internal standard obtained for the unknown should not differ by more than $\pm 20\%$ of the target ion ratio and acceptance criteria must be uniformly applied to all specimens within the batch [72]. The establishment of this 20% acceptance criteria for ion abundance ratios has been determined to be appropriate based on ion statistics [11-13,64]. Different ion ratio criteria cannot be applied to different specimens, calibrators, or controls within the batch.

C. Mass Spectral Match

To ensure adequate mass spectral match quality for laboratories utilizing full-scan acquisition, unknown mass spectra must be compared with reference spectra, and fit or match quality values must be computed. The laboratory should determine allowable limits of acceptability in accordance with laboratory studies and manufacturer's recommendations (e.g., 950 or greater out of a scale of 1,000). It is known that reference spectra in spectral libraries may at times differ from that obtained from analysis of actual specimens. This may be due to the type of instrument used, the particular algorithms used to generate a match, the number of ions used to establish a match, type of reagent gas, electron energy, or type of derivatization employed, among other factors. Therefore, the laboratory may consider establishing its own spectral library for analytes of interest, providing that manufacturer's specifications continue to be met and that generated spectra do not differ *significantly* from published reference sources.

In addition to spectral match requirements, the signal-to-noise ratio at the apex of the integrated peak of the analyte and its internal standard should be equal to or greater than a minimum of 10:1 at the assay's LOQ.

D. Quantitative Result

Regardless of the detection technique, in order to be designated as positive, the measured concentration of a specimen must be equal to or exceed the established assay cutoff concentration. Quantitative results around the cutoff must be truncated, rather than rounded up to the nearest whole number, so that the statistical bias is toward a "negative" result.

Specimens directed for GC/MS retest analyses are not subject to cutoff concentrations and are reported as reconfirmed if the concentration is equal to or greater than the LOD of the method. The specimen retest must also meet all other criteria for designating a positive test result. Quantitative reports should be provided only upon written request of the Medical Review Officer.

If the specimen concentration exceeds the linearity limits of the assay and the specimen was not diluted accordingly, the report must state: "the concentration of 'analyte' is greater than 'the established linearity limit'." Also, all criteria for designating a positive test result must be satisfied including chromatographic performance, ion ratios, and retention time data.

VI. MISCELLANEOUS FACTORS

A. Data Presentation

Although presentation of GC/MS data is highly variable among laboratories, printouts for all specimens (calibrators, controls, and positive and negative specimens) must include specimen identification information, total ion chromatogram illustrating entire acquisition window, individual selected ion chromatograms drawn to an approximate 0.5-minute window, mass spectrum (if applicable), spectral data including abundance and ion ratios or spectral match, retention time data, and concentration of analyte. In addition, data file name, date and time of injection, and MS operator name are highly desirable. Figures 2 and 3 illustrate typical GC/MS reports for confirmation of benzoylecgonine in urine using the Hewlett-Packard Mass Selective Detector and the Finnigan-MAT Ion Trap Mass Spectrometer, respectively. Further, a summary sheet presented in tabular format including injection sequence and specimen identification information, spectral data, and quantitative results should be prepared for ease of batch review (see Figure 4).

B. Dilution Protocols

Dilution protocols may be developed based upon the relationship of the immunoassay response and the quantitative result. Specimens may be diluted with GC/MS verified negative urine (or possibly purified water) prior to GC/MS confirmation in order to avoid carryover, prevent chromatographic overload, and to obtain acceptable chromatographic results. It is recommended that a quality control sample be diluted in the same manner as any routine specimen to assess the accuracy of the dilution.

A retest specimen may be tested by an immunoassay procedure in order to determine the need for dilution prior to GC/MS analysis. If dilution protocols are routinely applied by the laboratory, these procedures should be clearly described in the laboratory's SOP manual [72].

C. Reinjection of Extracts

Reinjection of specimen extracts may be necessary due to a failed injection or chromatographic overload. The laboratory must stipulate the number of acceptable reinjections (in regulated laboratories, more than one reinjection is generally considered unacceptable) and the maximum time after the end of the batch that reinjection is acceptable. If the reinjection is necessary due to column overload, the laboratory may elect to establish a policy whereby additional reconstitution solvent is added to the extraction vial prior to reinjection. However, such handling must be clearly recorded on laboratory documents, and the laboratory must establish minimum criteria for internal standard signal-to-noise and ion abundance to ensure that results continue to be reliable.

It is unacceptable to continue to reinject or reextract a specimen in order to "force" a negative or positive result. If a specimen fails acceptance criteria it can be reinjected one time and scheduled for re-extraction one time. However, if it continues to fail to meet criteria for the same reason, the specimen must be reported as negative. When reinjections are performed, it is necessary to reinject at least one standard and/or control in order to verify assay performance at the time of the reinjection. All initial and reinjection data, including failed or unacceptable data, must be maintained. Finally, retuning prior to reinjection is not permitted [72].

VII. REVIEW AND EVALUATION OF GC/MS DATA

All data must be thoroughly reviewed by a minimum of two individuals to verify compliance with the methods specified in the laboratory's procedure manual and to identify clerical and/or analytical errors. Batch acceptance criteria include within-range standards and controls and acceptable MS tune, chromatographic performance, ion abundance (adequate signal-to-noise ratio), and ion ratios or mass spectral match quality. Also, the laboratory may choose to monitor the consistency (reproducibility) of the ion abundance for the internal standard to ensure that it has been added appropriately, and at the correct concentration, to each calibrator, control, and unknown in confirmatory analyses. Furthermore, an acceptable calibration curve must be obtained, a lack of carryover must be demonstrated, and chain-of-custody documentation must be in order.

The SOP must also address the handling and reporting of results when duplicate extracts are assayed, or diluted and undiluted extracts are analyzed. Acceptance criteria for duplicates must specify minimum correlation of quan-

74

QUANTITATION REPORT FOR BE ON : 5972 - MS

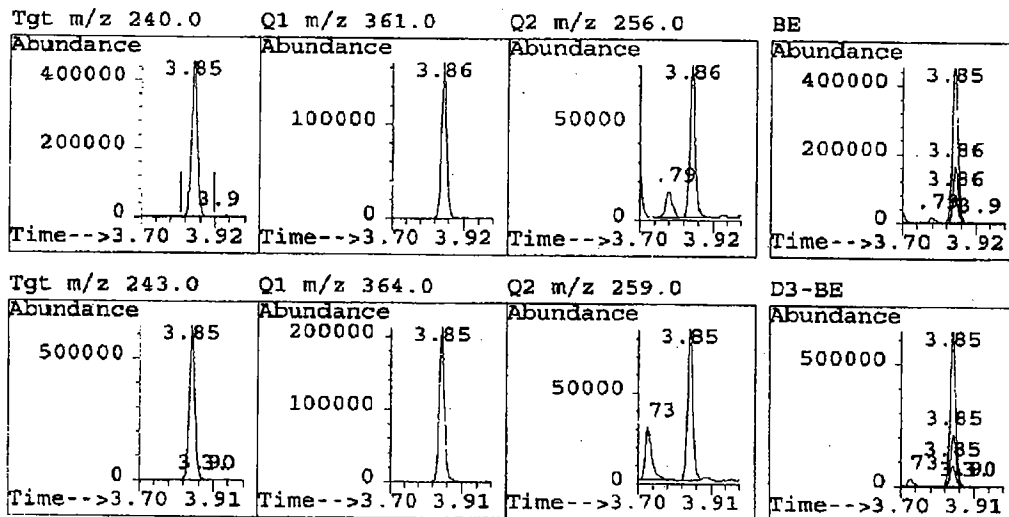
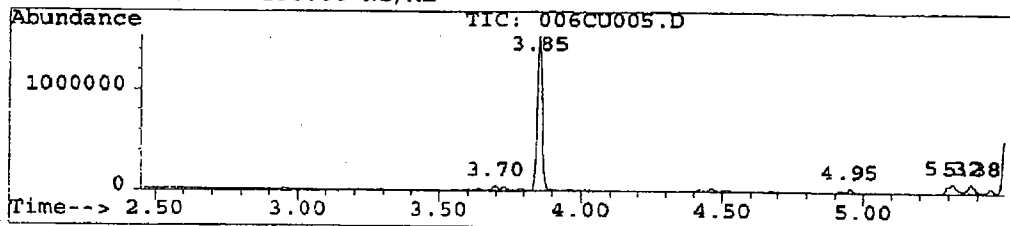
Data File : C:\HPCHEM\1\DATA\C5AUG96A.10A\006CU005.D
 Tune File Name : C:\HPCHEM\1\5972\ATUNE.U
 Tune Date : 5 Aug 96 9:42 am
 Acq Method Name : BEDRL.M Calib date : 05 Aug 96 1:24 pm
 Sample Name/Barcode : 150 NG/ML
 Acq date/Exp.Barcode : 5 Aug 96 11:43 am /

Retention Time 3.85 BE +/- 1.00% = 3.81 - 3.89 min
 Retention Time 3.85 D3-BE +/- 1.00% = 3.81 - 3.89 min
 R.R.T. = 1.001 Unknown target ion / ISTD target ion = 0.75

BE => 240.0 = 528009 361.0 = 183619 256.0 = 89775
 D3-BE => 243.0 = 707972 364.0 = 244509 259.0 = 95708

BE => 361.0/240.0 = 34.8 +/- 20.0% rel = 27.8 - 41.8
 BE => 256.0/240.0 = 17.0 +/- 20.0% rel = 13.6 - 20.4
 D3-BE => 364.0/243.0 = 34.5 +/- 20.0% rel = 27.6 - 41.4
 D3-BE => 259.0/243.0 = 13.5 +/- 20.0% rel = 10.8 - 16.2

Concentration = 144.84 ** CUTOFF CALIBRATOR **
 Cutoff limit = 150.00 NG/ML



75

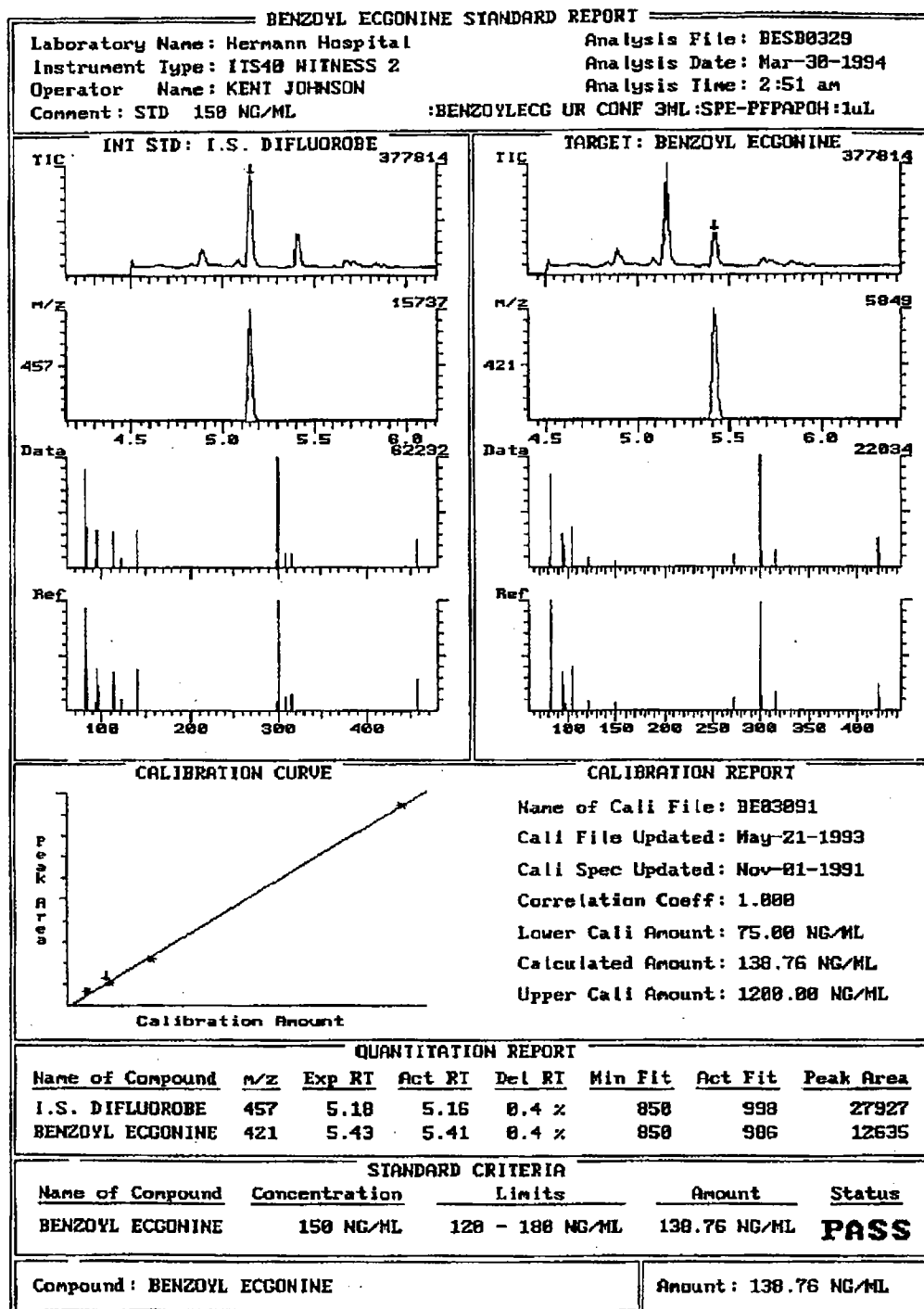


Figure 3. GC/MS report for confirmation of benzoylecgonine in urine using the Finnigan-MAT Ion Trap Mass Spectrometer. (Courtesy of Kent Johnson of Hermann Hospital: Houston, TX.)

Batch Summary Report **TOXICOLOGY LAB**

Batch Dir.: 05AUG96A.10A

Acquisition Date: 8/5/96 10:48 AM

Instrument Name: 5972 - MSD2

Drug Class: BENZOYLECGONINE

Operator: B. GOLDBERGER

Batch Name: 05AUG96A.10A

Original Method File: C:\HPCHEM\1\METHODS\BEDRL.M

Ion Ratio Range: +/-20%

Data Path: C:\HPCHEM\1\DATA\05AUG96A.10A

Retention Time Range: +/-1%

| Cutoff File Information | | | | | Quantitation Database | | | | | |
|-------------------------|---------------|--------------|------------|-------------|-----------------------|-----------|---------------|---------------|--------------|--------------|
| Compound Name | | Cutoff Conc. | File Name | Calc. Conc. | Sample Name | RT | Ratio 1 | Ratio 2 | ISTD Ratio 1 | ISTD Ratio 2 |
| BE | | 150 | 006CU005.D | 144.84 | 150 NG/ML | 3.85 | 34.78 | 16.98 | 34.54 | 13.52 |
| Run No. | Compound Name | Result | File Name | Calc. Conc. | Sample Name | Target RT | Target Ratio1 | Target Ratio2 | ISTD Ratio1 | ISTD Ratio2 |
| 1 | BE | POSITIVE | 001SP001.D | 155.98 | UNEXTRACTED STD | 3.85 | 34.94 | 16.80 | 33.97 | 13.24 |
| 2 | BE | Blank | 002BL000.D | 0.00 | SOLVENT BLANK | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 3 | BE | No ISTD | 003NE002.D | 0.00 | BLANK | 3.85 | 0.00 | 0.00 | 0.00 | 0.00 |
| 4 | BE | Good Neg. | 004NE003.D | 0.00 | BLANK + IS | 3.85 | 0.00 | 0.00 | 33.45 | 13.41 |
| 5 | BE | Calibrator | 005CA004.D | 74.45 | 75 NG/ML STD | 3.85 | 33.89 | 16.59 | 33.62 | 13.39 |
| 6 | BE | Cutoff | 006CU005.D | 144.84 | 150 NG/ML STD | 3.85 | 34.78 | 16.98 | 34.54 | 13.52 |
| 7 | BE | Calibrator | 007CA006.D | 1000.12 | 1000 NG/ML STD | 3.85 | 35.43 | 17.03 | 34.87 | 13.65 |
| 8 | BE | Blank | 008BL000.D | 0.00 | SOLVENT BLANK | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9 | BE | POSITIVE | 009SP007.D | 270.22 | R98-00001*5 | 3.86 | 35.82 | 17.05 | 35.34 | 13.49 |
| 10 | BE | Blank | 010BL000.D | 0.00 | SOLVENT BLANK | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 11 | BE | Negative | 011SP008.D | 135.59 | R98-00002*5 | 3.86 | 35.25 | 16.91 | 35.34 | 13.73 |
| 12 | BE | Blank | 012BL000.D | 0.00 | SOLVENT BLANK | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 13 | BE | Negative | 013SP009.D | 0.00 | QC NEGATIVE | 3.85 | 0.00 | 0.00 | 35.81 | 13.60 |
| 14 | BE | POSITIVE | 014SP010.D | 188.58 | QC POSITIVE | 3.85 | 36.11 | 17.40 | 34.66 | 13.98 |

End of Batch

Figure 4. GC/MS Batch Summary Review form.

titative results, usually $\pm 20\%$ if both results fall within the assay's linear range. In addition, both of the results must be equal to or greater than the mandated cutoff concentration, although use of the lowest value is recommended. The averaged results or a single value, diluted or undiluted, may be reported if both results meet acceptance criteria, and SOP requirements are consistently applied.

Review of specimen data should include comparison of the initial immunoassay response with the GC/MS result, an evaluation of chromatographic performance including presence of interfering or extraneous peaks, retention time, minimum ion abundance, ion ratios or mass spectral match quality, quantitation, extraction efficiency, potential for carryover, and chain-of-custody documentation.

REFERENCES

1. Armbruster DA, Tillman MD, Hubbs LM: Limit of detection (LOD)/Limit of quantitation (LOQ): Comparison of the empirical and the statistical methods exemplified with GC/MS assays of abused drugs; *Clin Chem* 40:1233; 1994.
2. Arnoux P, Morrison R: Drug analysis of biological samples. A survey of validation approaches in chromatography in the United Kingdom pharmaceutical industry; *Xenobiotica* 22:757; 1992.
3. Blanke RV: *Validation of the purity of standards*. Abbott Laboratories (Diagnostic Division): Abbott Park, IL; 1989.
4. Blau K, Halket JM (Eds): *Handbook of Derivatives for Chromatography*, 2nd ed; John Wiley & Sons: New York, NY; 1993.
5. Blau K, King GS (Eds): *Handbook of Derivatives for Chromatography*; Heyden & Son: London, U.K.; 1978.
6. Bonate PL: Concepts in calibration theory. Part I: Regression; *LC-GC* 10:310; 1992.
7. Bonate PL: Concepts in calibration theory. Part II: Regression through the origin — When should it be used? *LC-GC* 10:378; 1992.

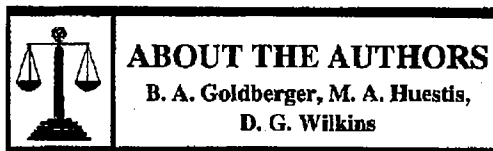
8. Bonate PL: Concepts in calibration theory. Part III: Weighted least-squares regression; *LC-GC* 10:448; 1992.
9. Boyd RK, Henion JD, Alexander M, Budde WL, Gilbert JD, Musser SM, Palmer C, Zurek EK: Mass spectrometry and good laboratory practices; *J Am Soc Mass Spectrom* 7:211; 1996.
10. Buick AR, Doig MV, Seal SC, Land GS, McDowal RD: Method validation in the bioanalytical laboratory; *J Pharm Biomed Anal* 8:629; 1990.
11. Baldwin R, Bethem RA, Boyd RK, Budde WL, Cairns T, Gibbons RD, Henion JD, Kaiser MA, Lewis DL, Matusik JE, Sphon JA, Stephany RW, Trubey RK: Report — 1996 ASMS Fall Workshop: Limits to confirmation, quantitation, and detection; *J Am Soc Mass Spectrom* 8:1180; 1997.
12. Cairns T, Siegmund EG, Stamp JJ: Evolving criteria for confirmation of trace level residues in food and drugs by mass spectrometry. Part I; *Mass Spectrom Rev* 8:93; 1989.
13. Cairns T, Siegmund EG, Stamp JJ: Evolving criteria for confirmation of trace level residues in food and drugs by mass spectrometry. Part II; *Mass Spectrom Rev* 8:127; 1989.
14. Cassidy R, Janoski M: Is your calibration curve linear? *LC-GC* 10:692; 1992.
15. Cembrowski GS, Carey RN: *Laboratory Quality Management*; ASCP Press: Chicago, IL; 1989.
16. Claeys M, Markey SP, Maenhaut W: Variance analysis of error in selected ion monitoring assays using various internal standards. A practical study case; *Biomed Mass Spectrom* 4:122; 1977.
17. Craft NE, Byrd GD, Hilpert LR: Preparation and certification of standard reference material 1507: 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid in freeze-dried urine; *Anal Chem* 61:540; 1989.
18. Dadgar D, Burnett PE, Choe MG, Gallicano K, Hooper JW: Application issues in bioanalytical method validation, sample analysis and data reporting; *J Pharm Biomed Anal* 13:89; 1995.
19. Davidian M, Carroll RJ, Smith W: Variance functions and the minimum detectable concentration in assays; *Biometrika* 75:549; 1988.
20. Dorschel CA, Ekmanis JL, Oberholtzer JE, Warren FV, Bidlingmeyer BA: LC detectors — Evaluation and practical implications of linearity; *Anal Chem* 61:951A; 1989.
21. Edwards AL: *An Introduction to Linear Regression and Correlation*, 2nd ed; WH Freeman and Company: New York, NY; 1976.
22. Ellerbe P, Long T, Welch MJ: The determination of amphetamine and methamphetamine in a lyophilized human urine reference material; *J Anal Toxicol* 17:165; 1993.
23. Ellerbe P, Tai SS-C, Christensen RG, Espinosa-Leniz R, Paule RC, Sander LC, Sniegoski LT, Welch MJ, White E: The certification of cocaine and benzoylecgonine in a human urine standard reference material; *J Anal Toxicol* 16:158; 1992.
24. ElSohly MA, Stanford DF, Sherman D, Shah H, Bernot D, Turner CE: A procedure for eliminating interferences from ephedrine and related compounds in the GC/MS analysis of amphetamine and methamphetamine; *J Anal Toxicol* 16:109; 1992.
25. Fenton J, Mummert J, Childers M: Hydromorphone and hydrocodone interference in GC/MS assays for codeine and morphine; *J Anal Toxicol* 18:159; 1994.
26. Foltz RL, Fentiman AF, Foltz RB: *GC/MS Analysis for Abused Drugs in Body Fluids* — NIDA Research Monograph 32; National Institute on Drug Abuse: Rockville, MD; 1980.
27. Garfield FM: *Quality Assurance Principles for Analytical Laboratories*; AOAC International: Arlington, VA; 1991.
28. Garland WA, Barbalas MP: Applications to analytic chemistry: An evaluation of stable isotopes in mass spectral drug assays; *J Clin Pharmacol* 26:412; 1986.
29. Green JM: A practical guide to analytical method validation; *Analyt Chem News Features* p 305A; May 1, 1996.
30. Grob K, Grob G, Grob K Jr: Testing capillary gas chromatographic columns; *J Chromatogr* 219:13; 1981.
31. Henion J, Covey T, Maylin G: Forensic mass spectrometry in equine drug testing; *Spectra* 10:21; 1985.
32. Hornbeck CL, Carrig JE, Czarny RJ: Detection of a GC/MS artifact peak as methamphetamine; *J Anal Toxicol* 17:257; 1993.
33. Inman EL, Frishmann JK, Jimenez PJ, Winkel GD, Persinger ML, Rutherford BS: General method validation guidelines for pharmaceutical samples; *J Chromatogr Sci* 25:252; 1987.
34. Joern WA: GC/MS assay of the marijuana carboxy metabolite: Urine interference with the dimethyl derivative; *J Anal Toxicol* 16:207; 1992.
35. Kaplan LA, Pesce AJ: *Clinical Chemistry: Theory, Analysis, and Correlation*; CV Mosby: St. Louis, MO; 1984.
36. Kaplan A, Szabo L: *Clinical Chemistry: Interpretation and Techniques*, 2nd ed; Lea & Febiger: Philadelphia, PA; 1983.
37. Karnes HT, March C: Precision, accuracy, and data acceptance criteria in biopharmaceutical analysis; *Pharmacol Res* 10:1420; 1993.
38. Karnes HT, Shiu G, Shah VP: Validation of bioanalytical methods; *Pharmacol Res* 8:421; 1991.
39. Knapp DR: Chemical derivatization for mass spectrometry; *Methods Enzymol* 193:314; 1990.
40. Knapp DR: *Handbook of Analytical Derivatization Reactions*; John Wiley & Sons: New York, NY; 1979.
41. Lang JR, Bolton S: A comprehensive method validation strategy for bioanalytical applications in the pharmaceutical industry. 1. Experimental considerations; *J Pharm Biomed Anal* 9:357; 1991.
42. Lang JR, Bolton S: A comprehensive method validation strategy for bioanalytical applications in the pharmaceutical industry. 2. Statistical analyses; *J Pharm Biomed Anal* 9:435; 1991.
43. Lawson AM, Gaskell SJ, Hjelm M: Methodological aspects on quantitative mass spectrometry used for accuracy control in clinical chemistry; *J Clin Chem Clin Biochem* 23:433; 1985.
44. Lawson GM: Defining the limit of detection and limit of quantitation as applied to drug abuse testing: Striving for a consensus; *Clin Chem* 40:1218; 1994.
45. Liu RH, Baugh DL, Allen EE, Salud SC, Fentress JG, Chadha H, Walia AS: Isotopic analogue as the international standard for quantitative determination of benzoylecgonine — Concerns with isotopic purity and concentration level; *J Forensic Sci* 34:986; 1989.
46. Liu RH, Foster G, Cone EJ, Kumar SD: Selecting an appropriate isotopic internal standard for gas chromatography/mass spectrometry analysis of drugs of abuse —

- Pentobarbital example; *J Forensic Sci* 40:983; 1995.
47. Liu RH, McKeehan AM, Edwards C, Foster G, Bensley WD, Langner JG, Wallia AS: Improved gas chromatography/mass spectrometry analysis of barbiturates in urine using centrifuge-based solid-phase extraction, methylation with d_5 -pentobarbital as internal standard; *J Forensic Sci* 39:1504; 1994.
 48. Long GL, Winefordner JD: Limit of detection; *Anal Chem* 55:712A; 1983.
 49. McNair HM: Method development in gas chromatography; *LC-GC* 11:794; 1993.
 50. Millard BJ: *Quantitative Mass Spectrometry*; Heyden & Sons: London, U.K.; 1978.
 51. Moffat AC, Jackson JV, Moss MS, Widdop B (Eds): *Clarke's Isolation and Identification of Drugs*, 2nd ed; The Pharmaceutical Press: London, U.K.; 1986.
 52. Moore JM: The application of chemical derivatization in forensic drug chemistry for GC and HPLC methods of analysis; *Forensic Sci Rev* 2:80; 1990.
 53. Nadkarni RA: The quest for quality in the laboratory; *Anal Chem* 63:675A; 1991.
 54. Needleman SB, Romberg RW: Limits of linearity and detection for some drugs of abuse; *J Anal Toxicol* 14:34; 1990.
 55. Nix ABJ, Wilson DW: Assay detection limits: Concept, definition, and estimation; *Eur J Clin Pharmacol* 39:203; 1990.
 56. Oppenheimer L, Capizzi TP, Weppleman RM, Mehta H: Determining the lowest limit of reliable assay measurement; *Anal Chem* 55:638; 1983.
 57. Paul BD, Past MR, McKinley RM, Foreman JD, McWhorter LK, Snyder JJ: Amphetamine as an artifact of methamphetamine during periodate degradation of interfering ephedrine, pseudoephedrine, and phenylpropanolamine: An improved procedure for accurate quantitation of amphetamines in urine; *J Anal Toxicol* 18:331; 1994.
 58. Pickup JF, McPherson K: Theoretical considerations in stable isotope dilution mass spectrometry for organic analysis; *Anal Chem* 48:1885; 1976.
 59. Podkowiak B-L, Repka ML, Smith ML: Interference of nitroindine in GC/MS confirmation of delta-9-THC-9-carboxylic acid in urine; *Clin Chem* 37:1305; 1991.
 60. Poole CF, Shuette S: *Contemporary Practice of Chromatography*; Elsevier: New York, NY; 1984.
 61. Provost LP, Elder RS: Interpretation of percent recovery data; *Am Lab* 15(12):57; 1983.
 62. Shewhart WA: *Statistical Method from the Viewpoint of Quality Control*; Dover Publications: New York, NY; 1986.
 63. Smith RV, Stewart JT: *Textbook of Biopharmaceutical Analysis*; Lea & Febiger: Philadelphia, PA; 1981.
 64. Sphon JA: Use of mass spectrometry for confirmation of animal drug residues; *J Assoc Off Anal Chem* 61:1247; 1978.
 65. Stevenson RY: An open letter to the food and drug administration; *Am Lab News Ed* 28(10):4; 1996.
 66. Tai SS-C, Christensen RG, Paule RC, Sander LC, Welch MJ: The certification of morphine and codeine in a human urine standard reference material; *J Anal Toxicol* 18:7; 1994.
 67. Thurman EM, Pedersen MJ, Stout RL, Martin T: Distinguishing sympathomimetic amines from amphetamine and methamphetamine in urine by gas chromatography/mass spectrometry; *J Anal Toxicol* 16:19; 1992.
 68. U.S. Department of Health and Human Services (Center for Drug Evaluation and Research, Food and Drug Administration): The draft guideline on validation of analytical procedures; *Fed Reg* 61:9315; 1996.
 69. U.S. Department of Health and Human Services (Center for Drug Evaluation and Research, Food and Drug Administration): *Reviewer Guidance — Validation of Chromatographic Methods*; 1994.
 70. U.S. Department of Health and Human Services (National Institute on Drug Abuse): Mandatory guidelines for federal workplace drug-testing programs; *Fed Reg* 53:11970; 1988.
 71. U.S. Department of Health and Human Services (National Laboratory Certification Program): Program Document #25; May 5, 1993.
 72. U.S. Department of Health and Human Services (Substance Abuse and Mental Health Services Administration): *Guidance Document for Laboratories and Inspectors*, Revised; August 29, 1994.
 73. U.S. Department of Health and Human Services (Center for Drug Evaluation and Research, Food and Drug Administration): *Guideline for Submitting Samples and Analytical Data for Methods Validation*; 1987.
 74. U.S. Department of Health and Human Services (Center for Drug Evaluation and Research, Food and Drug Administration): Draft guideline on validation of analytical procedures; *Fed Reg* 60:11260; 1995.
 75. Westgard JO: Better quality control through microcomputers; *Diagn Med* 61:741; 1982.
 76. Westgard JO, Barry PL: *Cost-Effective Quality Control: Managing The Quality and Productivity of Analytical Processes*; AACC Press: Washington, DC; 1986.
 77. Westgard JO, Barry PL, Hunt MR, Groth T: A multi-rule Shewhart chart for quality control in clinical chemistry; *Clin Chem* 27:493; 1981.
 78. Williams DR: An overview of test method validation; *BioPharm Manuf* 0:34; 1987.
 79. Wu AHB, Ostheimer D, Cremese M, Forte E, Hill D: Characterization of drug interferences caused by coelution of substances in GC/MS confirmation of targeted drugs in full-scan and selected-ion monitoring modes; *Clin Chem* 40:216; 1994.
 80. Wu Chen NB, Cody JT, Garriott JC, Foltz RL, Peat MA, Schaffer MI: Report of the 1988 Ad Hoc Committee on Forensic GC/MS: Recommended guidelines for forensic GC/MS procedures in toxicology laboratories associated with offices of medical examiners and/or coroners; *J Forensic Sci* 35:236; 1990.
 81. Yinon J: *Forensic Mass Spectrometry*; CRC Press: Boca Raton, FL; 1987.

ADDITIONAL READINGS

1. Aarons L: Effect of experimental design on assay calibration; *Analyst* 106:1249; 1981.
2. Bonate PL: Concepts in calibration theory. Part IV: Prediction and confidence intervals; *LC-GC* 10:531; 1992.
3. Burlingame AL, Boyd RK, Gaskell SJ: Mass spectrometry; *Anal Chem* 68:599R; 1996.
4. Chamberlain J: *Analysis of Drugs in Biological Fluids*, 2nd ed; CRC Press: Boca Raton, FL; 1995.

5. Freeman RR (Ed): *High Resolution Gas Chromatography*, 2nd ed; Hewlett-Packard: Wilmington, DE; 1981.
6. National Committee for Clinical Laboratory Standards: *Internal Quality Control Testing — Principles and Definitions, Approved Guidelines (C24-A)*; Villanova, PA; 1991.
7. Price P: Standard definitions of terms relating to mass spectrometry — A report from the Committee on Measurements and Standards of the American Society for Mass Spectrometry; *J Am Soc Mass Spectrom* 2:336; 1991.
8. Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Viswanathan CT, Cook CE: Analytical methods validation — Bioavailability, bioequivalence, and pharmacokinetic studies; *Eur J Drug Metab Pharmacokin* 16:249; 1991.
9. Taylor JK: *Quality Assurance of Chemical Measurements*; Lewis: Chelsea, MI; 1987.



Bruce A. Goldberger received a B.A. degree in zoology from Drew University (Madison, NJ) and M.S. and Ph.D. degrees in forensic toxicology from the School of Medicine at the University of Maryland in Baltimore (Baltimore, MD). Dr. Goldberger is currently an assistant professor and is director of toxicology in the Diagnostic Referral Laboratories of the Department of Pathology, Immunology, and Laboratory Medicine in the College of Medicine at the University of Florida (Gainesville, FL).

Dr. Goldberger is certified as a Toxicological Chemist by the National Registry in Clinical Chemistry and a Forensic Toxicology Specialist by the American Board of Forensic Toxicology. Dr. Goldberger was an assistant toxicologist and laboratory manager for the State of Maryland's Office of the Chief Medical Examiner and a toxicologist with the National Center for Forensic Science (Baltimore, MD). Dr. Goldberger's studies have included the analysis of alcohol in breath and the measurement of therapeutic and abused drugs in biological tissues. He has published numerous articles related to forensic toxicology and is co-editor (with Ray H. Liu of the University of Alabama at Birmingham) of the *Handbook of Workplace Drug Testing* (American Association for Clinical Chemistry Press: Washington, DC; 1995).

In recognition of his achievements in research in forensic toxicology, Dr. Goldberger was awarded the first annual Irving Sunshine Award from the Toxicology Section of the American Academy of Forensic Sciences (AAFS) in 1988. In addition, he was the 1994 recipient of the American Association for Clinical Chemistry's Outstanding Scientific Achievements by a Young Investigator Award. Dr. Goldberger is an active member of AAFS, American Association for Clinical Chemistry (AACC), National Safety Council's Committee on Alcohol and Other Drugs, and the Society of Forensic Toxicologists (SOFT).

Marilyn A. Huestis received a bachelor's degree in biochemistry from Mount Holyoke (South Hadley, MA), a master's degree in clinical chemistry from the University of New Mexico (Albuquerque, NM), and a doctoral degree in toxicology from the University of Maryland in Baltimore (Baltimore, MD), where she received a full research scholarship. Currently, Dr. Huestis is a senior research scientist at the Addiction Research Center (ARC), National Institute on Drug Abuse (NIDA), National Institute of Health (NIH), where her research interests include: (a) the pharmacokinetics and pharmacodynamics of drugs of abuse in humans, (b) evaluation of the effects of drugs on behavior including correlations of blood levels of drugs and concurrent changes in performance, (c) evaluation and validation of analytical techniques for detection of drugs of abuse in different biological fluids, and (d) medication development projects including the use of buprenorphine as a pharmacotherapeutic agent in opioid dependence. Dr. Huestis is interested in evaluating diverse routes of drug administration and determining the effect of different routes on the abuse liability of drugs, effects on performance, and on the onset, peak, and duration of action of the resultant effects.

Dr. Huestis has been working in the fields of forensic and analytical toxicology and clinical chemistry for more than 20 years. She was chief toxicologist of Nichols Institute's San Diego laboratory from 1983 to 1988, where she directed the emergency toxicology, therapeutic drug monitoring, analytical toxicology, and urine drug-testing operations. Dr. Huestis has published several important papers in the areas of marijuana pharmacokinetics and pharmacodynamics, in the evaluation and validation of immunoassays, and on the use of alternate testing matrices for drug analysis including hair, saliva, and sweat. She was nominated for the research staff fellow award of the ARC for her studies on the pharmacokinetics and pharmacodynamics of marijuana use. She has also been awarded the AAFS Irving Sunshine Award for outstanding research in forensic toxicology in 1992, and was elected to the Phi Kappa Phi Honor Society for outstanding academic achievement.

Goldberger, Huestis, and Wilkins • Quality Control Procedures for GC/MS Forensic Urine Drug Testing

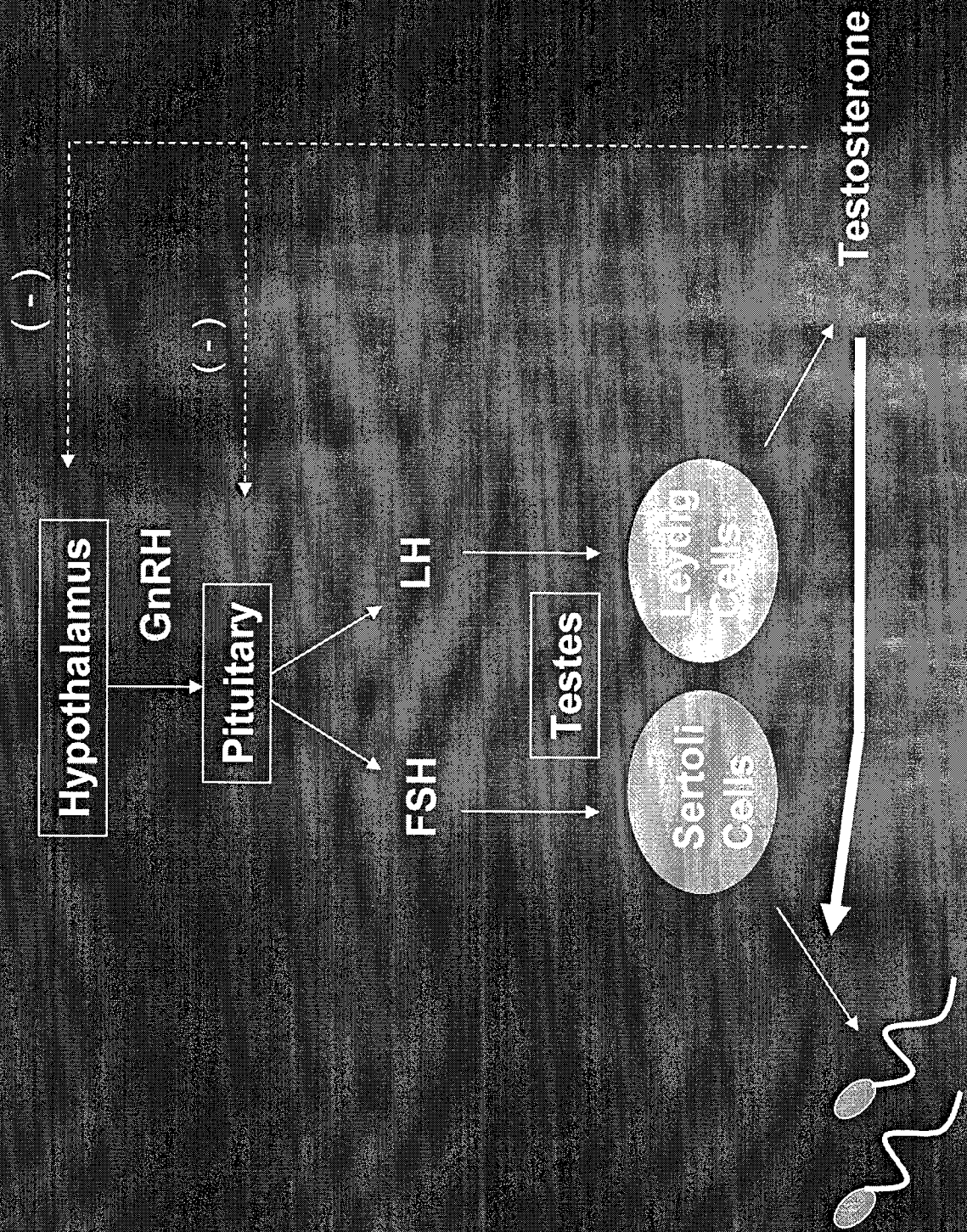
Dr. Huestis is on the Board of Directors of SOFT and currently serves as secretary of this organization. She also is program chair for the Toxicology Section of AAFS, is an active member and former secretary of the California Association of Toxicologists (CAT), serves on the Therapeutic Drug Monitoring and Clinical Toxicology Committee of AACC, and is a member of the International Association of Forensic Toxicology (TIAFT). Dr. Huestis was co-chair of the joint TIAFT/SOFT Joint Congress held in Tampa, FL in 1994.

Diana G. Wilkins received B.S. (1985), M.S. (1988), and Ph.D. (1991) degrees from the University of Utah (Salt Lake City, UT). She is currently assistant director of the Center for Human Toxicology at the University of Utah Health Sciences Center. She concurrently holds a faculty position as research assistant professor in the Department of Pharmacology and Toxicology, College of Pharmacy at the University of Utah.

Previously, Dr. Wilkins served as the quality control/quality assurance manager and senior toxicologist at the Center for Human Toxicology. Her postdoctoral education included training in toxicology and pharmacology, with particular emphasis on drugs of abuse. She currently participates in teaching undergraduate and graduate courses in clinical and analytical toxicology, pharmacology, and statistics for pharmacologic research at the University of Utah. Her research interests include investigation of the disposition of drugs of abuse in human hair and other biological matrices, as well as applications of mass spectrometry in biomedical research. She has obtained a research grant from NIDA to study the application of hair analysis to the monitoring of treatment compliance.

Dr. Wilkins is a laboratory inspector for the National Laboratory Certification Program under the auspices of the Department of Health and Human Services (HHS) and a laboratory assessor for the Standards Council of Canada. Dr. Wilkins was a member of the Drug Testing Advisory Board of the HHS Division of Workplace Performance for the period of 1994-1997. She has also served as an ad hoc member of several NIDA study sections. She belongs to several scientific associations, including AAFS, SOFT, TIAFT, CAT, and the Association for Women in Science.

Hormonal Control of Testicular Function



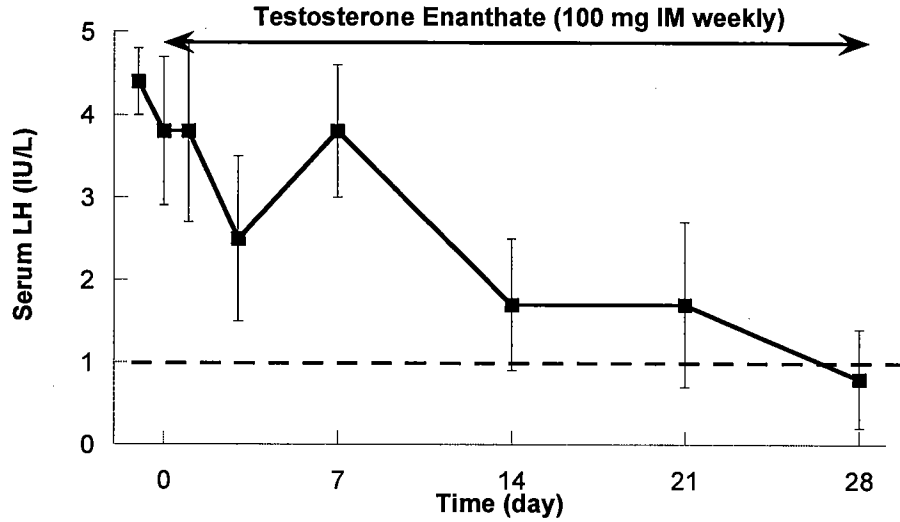


Figure. Serum luteinizing hormone (mean \pm SEM) prior to and during the administration of testosterone enanthate (100 mg IM q week) to seven normal men. The dotted line represents the lower limit of the normal range. Note that 4 weeks of testosterone administration are required to suppress serum luteinizing hormone release from the pituitary to abnormally low levels (Amory et al, manuscript in preparation).

Effect of multiple oral doses of androgenic anabolic steroids on endurance performance and serum indices of physical stress in healthy male subjects

Norbert Baume · Yorck Olaf Schumacher ·
Pierre-Edouard Sottas · Carlo Bagutti ·
Michel Cauderay · Patrice Mangin · Martial Saugy

Accepted: 4 July 2006 / Published online: 3 August 2006
© Springer-Verlag 2006

Abstract Anabolic androgenic steroids (AAS) are doping agents that are mostly used for improvement of strength and muscle hypertrophy. In some sports, athletes reported that the intake of AAS is associated with a better recovery, a higher training load capacity and therefore an increase in physical and mental performances. The purpose of this study was to evaluate, the effect of multiple doses of AAS on different physiological parameters that could indirectly relate the physical state of athletes during a hard endurance training program. In a double blind settings, three groups ($n = 9, 8$ and 8) were orally administered placebo, testosterone undecanoate or 19-norandrostenedione, 12 times during 1 month. Serum biomarkers (creatine kinase, ASAT and urea), serum hormone profiles (testosterone, cortisol and LH) and urinary catecholamines (noradrenalin, adrenalin and dopamine) were evaluated during the treatment. Running performance was assessed before and after the intervention phase by

means of a standardized treadmill test. None of the measured biochemical variables showed significant impact of AAS on physical stress level. Data from exercise testing on submaximal and maximal level did not reveal any performance differences between the three groups or their response to the treatment. In the present study, no effect of multiple oral doses of AAS on endurance performance or bioserum recovery markers was found.

Keywords Anabolic androgenic steroids · Doping · Recovery · Endurance training · Running

Introduction

The main aim of each professional or amateur athlete is to reach his maximal performance. To succeed, athletes need to balance between training loads (intensity and volume) and recovery periods which allow them to optimise their physiological and psychological capacities. Thus, training programs and specially recovery phases have to be well defined in order to avoid a prolonged imbalance that could lead to overreaching and/or overtraining (Halsen and Jeukendrup 2004; Hug et al. 2003; Kuipers and Keizer 1988; Lehmann et al. 1993; Petibois et al. 2002). In increasing the recovery capacities of the organism, the training volumes and intensities can be amplified thus reducing the risk to reach an overtraining state.

Recently, in endurance sports like cycling and athletics, some athletes reported anonymously the use of anabolic steroids, like testosterone and nandrolone, as an efficient exogenous aid to recover more rapidly following intense physical efforts (Hartgens and Kuipers 2004).

N. Baume (✉) · P. E. Sottas · P. Mangin · M. Saugy
Laboratoire Suisse d'Analyse du Dopage,
Institut Universitaire de Médecine Légale,
Ch. des Croisettes 22, 1066 Epalinges, Switzerland
e-mail: norbaume@gmail.com

Y. O. Schumacher
Abtlg. Sportmedizin, Medizinische Universitätsklinik
Freiburg, Hugstetter Strasse 55, 79106 Freiburg, Germany

C. Bagutti
Centre Médical Vidy-Med, Rte de Chavannes 11,
1000 Lausanne 23, Switzerland

M. Cauderay
Hôpital Riviera, Site du Samaritain, Bd. Paderewski 3,
1800 Vevey, Switzerland

The discovery of testosterone has given rise to the synthesis of multiple anabolic steroids (Kenyon et al. 1938). Rapidly, these products as well as testosterone have been used for doping purposes even if their performance-enhancing properties were largely inconclusive for many years (Haupt and Rovere 1984). Nevertheless, since 1980 some authors reported evidences that anabolic androgenic steroids (AAS) are effective in increasing muscle mass and improving strength (Bhasin et al. 1996; Friedl et al. 1991; Hartgens and Kuipers 2004) but the research done on the recovery rate in humans is too limited to draw definite conclusions yet. As recuperation is a physical and mental concept that is quite difficult to measure directly, the few research done in this context studied indirect parameters, like heart rate and serum lactate levels, creatinine, creatine kinase (CK) and aspartate aminotransferase (ASAT), associated with recovery time. Based on these criteria, some authors reported beneficial effects of AAS (Boone et al. 1990; Keul et al. 1976; Rozenek et al. 1990; Tamaki et al. 2001) whereas others found no consequence of AAS on recuperation (Jakob et al. 1988; Urhausen et al. 1989). Through experiments on birds, Alway and Starkweather (1993) suggested that the increased rate of protein synthesis due to AAS administration could be at the origin of a faster recovery from training load. An extrapolation was done from birds to humans without any scientific proofs.

As AAS are still at the origin of the major adverse analytical findings in the world antidoping laboratories (WADA 2005), the aim of the present study was to investigate more precisely the effect of multiple oral doses of testosterone undecanoate, 19-norandrostenedione or placebo on physical stress in male volunteers during a period of endurance training. Regarding ergogenics effects, the two investigated AAS can not be compared with others main AAS like metandienone or stanozolol which effects on muscle development and lean body mass have been clearly proven (Broeder 2003; Hartgens and Kuipers 2004).

Furthermore, we hypothesised that exogenous AAS intake could affect several blood and urinary parameters. Then some biological markers were analysed through the whole protocol in order to detect a potential typical profile of AAS doped athletes versus placebo. Hypothalamus-pituitary axis hormones (testosterone, cortisol, LH, hCG and SHBG) were measured to assess the effect of the administered AAS on blood hormones profile. Cardiac and muscular markers (Troponin I, Myoglobin, ALAT and Creatine Kinase) as well as inflammatory cytokine (IL-6) and catecholamines have been assessed to evaluate the

physiological effects of strenuous exercise in treated and placebo volunteers.

At the same time, running performances were measured through treadmill tests at the beginning and at the end of the protocol in order to put forward a positive global effect of testosterone and/or 19-norandrostenedione on physical adaptations to training.

Materials and methods

Selection of volunteers

The study was conducted at the Swiss Antidoping Laboratory, in Lausanne (Switzerland), after the approval of the protocol by the ethics committee from the Faculty of Medicine (University of Lausanne). Participants were healthy male volunteers from Caucasian origin, aged between 20 and 30, mainly from the Institute of Sports Sciences and Physical Education (University of Lausanne, Switzerland). All subjects practiced sports on a regular base with weekly training times ranging within 4 and 12 h. Thirty volunteers provided informed consent for the participation in the study. These students were not physically dependant on drugs and were instructed not to take any nutritional supplements or steroids 1 year before and during the study, which could affect their metabolism and steroid profile. Volunteers who were prone to hormonal dysfunction, cardiovascular disease, or have used controlled substances or any medication known to affect steroid metabolism were excluded from the study. Participants were evaluated medically (anamnesis and ECG at rest) and psychologically at the beginning of the study, before the treatment period, and at the end of the study in order to monitor their health. The anthropometrical characteristics of the participants as well as their physical level are summarized in Table 1. The fat percentage was determined with a four site (triceps, biceps, subscapular and suprailiac) skinfold measurement (Pongchaiyakul et al. 2005).

Exercise testing

The trial was divided in three phases. During the first part, all subjects performed a medical inclusive test and a running exercise test 1 month prior to the beginning of the treatment period. The exercise test was done on a ELM2 treadmill (Woodway, Weil am Rhein, Germany) where velocity was increased incrementally until subjective exhaustion of the tested subject. The initial workload was 6 km/h and running velocity was increased by 2 km/h at 3 min intervals. Blood lactate

Table 1 Mean anthropometrics values and physical performance (\pm SD) of the three groups at the beginning and at the end of the study

| | Placebo | | 19-Norandrostenedione | | Testosterone | |
|-----------------------------|-----------------|-----------------|-----------------------|-----------------|-----------------|-----------------|
| | Before | After | Before | After | Before | After |
| Age (years) | 24.2 \pm 2.3 | | 22.9 \pm 2.0 | | 24.1 \pm 1.6 | |
| Weight (kg) | 75.7 \pm 8.4 | 74.2 \pm 8.0 | 77.3 \pm 7.6 | 77.5 \pm 6.9 | 76.6 \pm 9.5 | 76.2 \pm 9.1 |
| Height (cm) | 182.4 \pm 4.5 | 182.4 \pm 4.5 | 183.4 \pm 5.9 | 183.4 \pm 5.9 | 182.7 \pm 6.6 | 182.7 \pm 6.6 |
| Fat mass (%) | 12.6 \pm 3.6 | 12.4 \pm 3.6 | 12.6 \pm 1.7 | 12.7 \pm 1.3 | 12.5 \pm 3.4 | 12.5 \pm 2.7 |
| Free fat mass (kg) | 66.0 \pm 6.1 | 64.9 \pm 5.6 | 67.5 \pm 6.8 | 67.6 \pm 6.1 | 66.9 \pm 6.8 | 66.6 \pm 6.9 |
| Body mass index | 20.7 \pm 2.0 | 20.3 \pm 1.8 | 21.0 \pm 1.5 | 21.1 \pm 1.3 | 21.0 \pm 2.2 | 20.8 \pm 2.0 |
| Running speed at IAT (km/h) | 13.3 \pm 1.9 | 14.4 \pm 1.8 | 12.7 \pm 1.5 | 14.0 \pm 0.7 | 13.6 \pm 0.7 | 14.7 \pm 1.2 |

concentrations at the different running velocities was measured with a Lactate Pro (Arkray, Kyoto, Japan) in capillary blood obtained during a 20 s-break after each stage from the hyperaemised earlobe. Heart rate was registered continuously using surface ECG during the last 30 s of each step of the protocol.

Training program

A special software (Ergonizer, Freiburg, Germany) was used for investigator-independent calculation of lactate threshold based on an equalizing SPLINE interpolation procedure (Roecker et al. 1998). As demarcations of intensity, heart rate at the lactate threshold (LT) and the so-called individual anaerobic threshold (IAT) were determined. The LT was taken from the original definition introduced by Wassermann et al. (1999) at the workload that corresponds to the start of the blood lactate concentration increase. IAT was set as the point at a net lactate increase of 1.5 mmol/l above LT (Dickhuth et al. 1991). Four individual training zones for each volunteer were defined based on the heart rate at the IAT as proposed by Roecker et al. (2002). Zone 1 (83–100% of LT) was used as exercise intensity for recovery or warm-up training. Zone 2 and 3 (100% of LT–95% of IAT for zone 2 and 95–101% of IAT for zone 3) determined the training intensity to improve aerobic performance. Training in zone 4 (99–107% of IAT) was used to increase the lactate tolerance (Roecker et al. 1997).

In order to level the performance of all participants, volunteers had to run four times a week at a low intensity included in the first or second training zone, 2 weeks before the first treatment. To manage their training during the protocol, a heart rate monitor (Fitwatch, Sigma Sport, Germany) was lent to the subjects.

Study medication

The second part of the study consisted in the oral intake of 12 placebo, 19-norandrostenedione or testos-

terone undecanoate pills on Monday, Wednesday and Friday between 6.30 and 8 am for 4 weeks (day 1–28). The subjects were randomly distributed in the three groups of ten individuals. The pills were composed of 300 mg of mannitol for placebo group (P group), 100 mg of 19-nor-4-androstenedione and 127 mg of mannitol for 19-norandrostenedione group (N group), 80 mg of testosterone undecanoate and 115 mg of mannitol for testosterone group (T group). Testosterone undecanoate (Dynapharm Distribution, Meyrin, Switzerland) and 19-nor-4-androstenedione (Sigma, St. Louis, MO, USA) were analytical tested by the Pharmacy of the University Hospital (CHUV, Lausanne, Switzerland) that also prepared the pills.

During the same time period, the volunteers were subjected to a strict individual training program (Fig. 1) aimed at improving endurance performance and causing fatigue and designed by expert staff. Meanwhile, all the subjects had to fill in the short version of POMS (Profile of Mood State) in order to assess the psychological profiles (data submitted for publication).

Spot urines were collected before the first pill intake, during the second week of the training period before and after the running sessions, on day 24 before treatment, 4, 8 and 24 h after and 13 days after the last pill intake (Fig. 1). Urines were kept at 4°C until their delivery to the laboratory and then each urine sample was divided into 20 ml flasks and stored at –20°C until extraction and analysis at the laboratory.

Blood analysis

Blood collections consisted in three tubes, one EDTA 2.7 ml and two serum 4.9 and 5.5 ml (Sarstedt AG & Co., Nümbrecht, Germany) withdrawn before the administration of the pill on day 1, 5, 8, 10, 12, 19, 26 and 29 in fasting conditions. The last tube was collected on day 38, i.e. 10 days after the end of the training program. The tubes were kept at 4°C (maximum 2 h) until the arrival at the laboratory. Hemograms were analysed on the EDTA tubes with a Sysmex XT 2000i

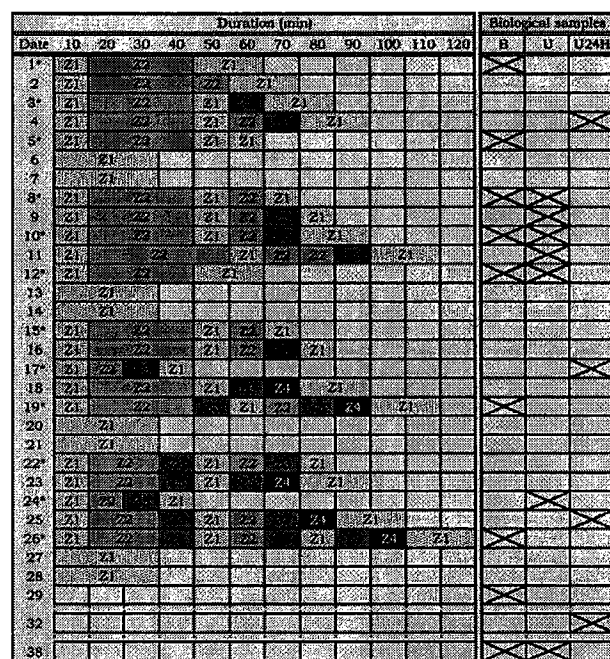


Fig. 1 Schematic representation of the main part of the protocol. The pill intakes are shown by the *asterisks*. The daily training program is detailed with the different training zones (*box with dots*, zone 1; *light grey shaded box*, zone 2; *dark grey shaded box*, zone 3 and *black box*, zone 4). The schedule of sampling is depicted on the *right side* (B, blood; U, urine; U24H: urines during 24 h)

(Sysmex Corporation, Kobe, Japan). These samples were then centrifuged 10 min at 1,700 g and plasma distributed in 3 ml polypropylene tubes and stored at -20°C until further analysis.

Serum tubes were directly centrifuged 10 min at 1,700 g and the plasma collected in 3 ml polypropylene tubes and stored at -20°C until analysis. Serum endocrinology parameters (cortisol, testosterone, LH, hCG, SHBG), muscle and cardiac biomarkers (Creatine Kinase, Troponin I, Myoglobine) and inflammatory markers (Interleukine-6) were measured with an Immulite 2000 (DPC Diagnostic Products Corporation, Los Angeles, CA, USA).

Aspartate, alanine aminotransferase (ASAT, ALAT) and urea were obtained from Dade Behring Diagnostics (Düdingen, Switzerland), runned on Dimension® RXL.

Catecholamines analysis

The subjects were asked to collect all their urines during 24 h on day 4, 17, 25 and 32 (U24H, see Fig. 1). Ten millilitres of HCl 5 N (Merck, Darmstadt, Germany) were added in the 3 l containers (Becton Dickinson BD, Franklin Lakes, USA) to stabilize the urines. Two

aliquots were taken in 10 ml urine monovette (Sarstedt AG & Co., Nümbrecht, Germany) and kept frozen at -20°C until analysis. Adrenalin, noradrenalin and dopamine were measured using high performance liquid chromatography (HPLC) with fluorescence detection (LC 240, Perkin Elmer, Boston, MA, USA).

Statistical analysis

All statistical analysis were performed on Matlab® version 6.1.0, with Statistics Toolbox version 3.0. Since distributions of the concentrations under study here are not necessarily normally distributed, a non-parametric one-way analysis of variance (Kruskal–Wallis ANOVA) was used for comparing means or medians between two groups of data. A *t*-test was also used to compare the sample average to a given constant. A significance level of $P = 0.05$ was considered.

Results

General considerations

Thirty male individuals were recruited to take part to this trial. Because of the vigorous training program imposed on the volunteers, five of them retired from the study due to physical problems (articulations and muscles soreness). The values measured on these individuals were not included in the analysis. The final repartition of subjects was 9, 8 and 8 individuals for P, T and N group, respectively. The anthropometrics values (Table 1) showed no statistical difference ($P > 0.05$) between the three groups before and at the end of the protocol.

The spot urines collected during the protocol were analysed for the detection of the endogenous urinary steroids. Isotope ratio mass spectrometry and gas chromatography coupled to mass spectrometry were used to detect variations of the testosterone metabolites compounds (Baume et al. 2006). Nandrolone metabolites (19-norandrosterone and 19-noretiocholanolone) stemming from the metabolism of 19-norandrostenedione (Bricout and Wright 2004) were also quantified in order to support previously published results (Baume et al. 2004) on the nandrolone metabolites pharmacokinetics (manuscript in preparation).

Physical performance

Three weeks before the first study medication, speed, heart rate, serum lactate concentration and exhaustion were determined during standardized treadmill test.

No statistical difference was found between the groups except for lactate I_{AT} and maximal speed between N and T groups ($P_{NT} = 0.02$ and 0.03). Statistical analysis were done on the same parameters established following a second treadmill test performed 10 days after the end of the training program. The results revealed no difference between the three groups ($P > 0.05$).

To put forward a potential beneficial effect of numerous oral doses of AAS on performance, the same physiological parameters were used. The intra-group differences between the post and the pre-treatment treadmill test values were calculated. As depicted in Fig. 2, a significant increase was observed for maximal speed ($\Delta_{\text{max speed}}$) in all groups (t -test for mean = 0; P_P, P_N and $P_T < 0.05$) and for the speed at the I_{AT} ($\Delta_{\text{speed } I_{AT}}$) for the N group. A noteworthy decrease was noted for the serum lactate concentration at the I_{AT} ($\Delta_{\text{lactate } I_{AT}}$) for P and N groups (P_P and $P_N < 0.05$).

Blood analysis

Several serum endocrinology parameters were measured. The time course of cortisol, total testosterone,

LH, T/LH and T/cortisol ratios along the protocol is depicted in Fig. 3. Comparison (Kruskal–Wallis analysis of variance) of values from the first and the last blood samples showed a significant decrease of cortisol for P and N groups ($P = 0.0007$ and $P = 0.0046$) and of total testosterone for the three groups ($P = 0.0041$, $P = 0.046$ and $P = 0.0063$, for P, N and T group, respectively). The T/LH ratio did not vary along the entire study (Kruskal–Wallis ANOVA; $P > 0.05$) but the T/Cortisol ratio increased significantly for the P group ($P = 0.01$). The first value measured before the beginning of treatment was compared to a group of values composed of the samples 2–8 (days 5, 8, 10, 12, 19, 26 and 29; i.e. during the treatment). No significant variation was noted for all the investigated parameters. The results of hCG analysis were mainly below the detection limit of the test used and are therefore not of use for any interpretation.

Cardiac biomarkers were also measured on all blood samples. Figure 4 shows the variations of creatine kinase. Additionally, the time course of ASAT and urea is depicted in same figure. The statistical analysis did not reveal any intragroup difference (Kruskal–Wallis ANOVA; $P > 0.05$). It has to be noticed that the CK data are expressed in ng/ml which is uncommon and

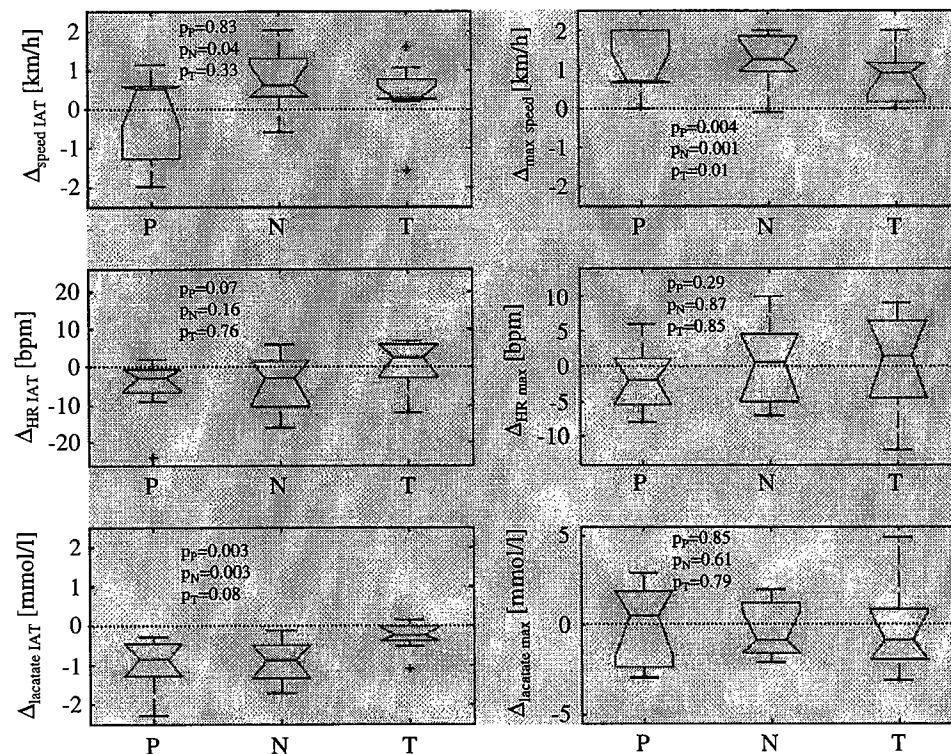


Fig. 2 Individual differences (Δ) between post and pre-treatment speed, heart rate (HR) and lactate values at the individual anaerobic threshold (I_{AT} , left part of the chart) and at exhaustion (max, right part of the chart) for the three groups placebo, 19-norandrostenedione and testosterone. P values indicate the probability of

a significant variation ($P < 0.05$) due to the administered product (i.e. P_T for the testosterone group). The boxes contain 25–75% of the values and the bars illustrate the 95% confidence interval. The median is represented by the line in the boxes. The crosses are the outliers

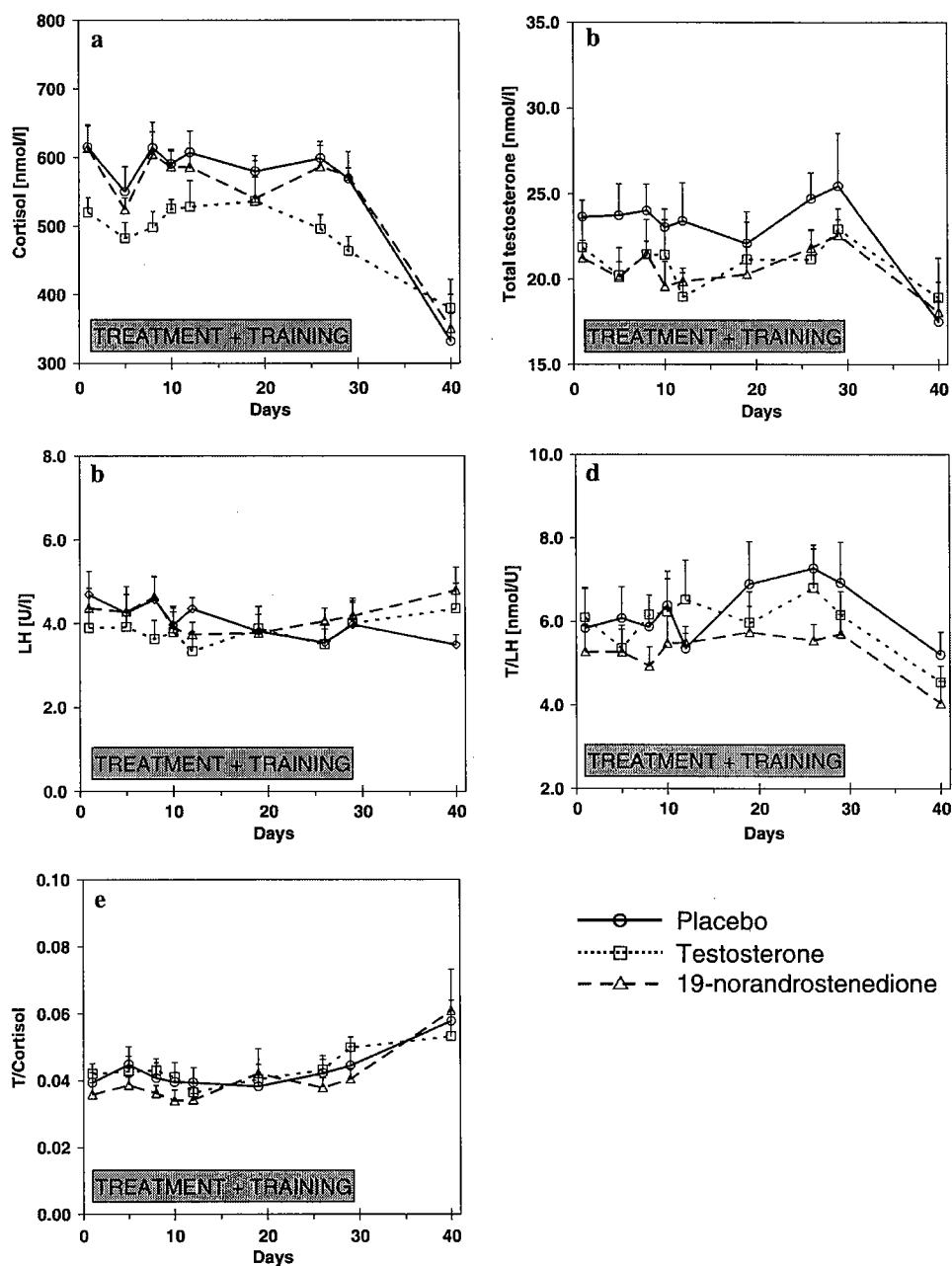


Fig. 3 Values of cortisol (a), total testosterone (b), LH (c), T/LH ratio (d) and T/Cortisol ratio (e) for the placebo (open circles, unbroken line), testosterone (open square, dotted line) and 19-no-

randrostenedione (open triangle, broken line) groups. Values are means \pm SEM. The training and treatment period (28 days, see Fig. 1) is indicated by the shadowed boxes

due to the measurement method. To put the data into perspective, a reference range study revealed a median value of 0.9 ng/ml, an upper 97.5th percentile of 2.6 ng/ml, and an upper 99 percentile of 4.8 ng/ml (Immulate 2000 CK-MB procedure, DPC, CA, USA).

As hCG, the detection of troponine I could not be used because of concentrations ranging below the detection limit of the test used. Myoglobin values remained invariant, the data are therefore not shown.

The blood parameters (full blood count including Hct, Hb, ...) measured on the EDTA tubes revealed no significant variation throughout the trial ($P > 0.05$) (data not shown).

Urinary catecholamines

Four times during the protocol, the volunteers had to collect their urines during 24 h (Fig. 1). The three

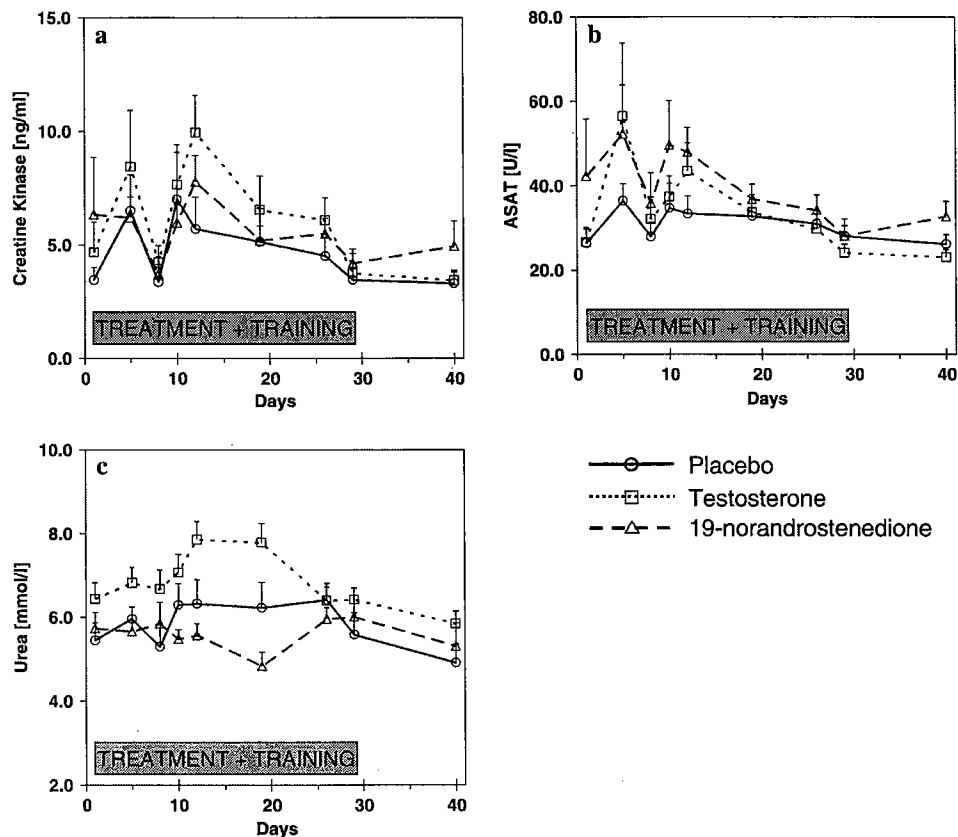


Fig. 4 Values of creatine kinase (a), ASAT (b) and urea (c) for the placebo (open circles, unbroken line), testosterone (open square, dotted line) and 19-norandrostenedione (open triangle,

broken line) groups. Values are means \pm SEM. As in Fig. 3, the training and treatment phase is indicated

major catecholamines, noradrenalin, adrenalin and dopamine were measured. The mean values of the four collection days are depicted in Fig. 5. The values are within the normal range (noradrenalin: 20–80 $\mu\text{g}/24\text{ h}$; adrenalin: 2–20 $\mu\text{g}/24\text{ h}$; dopamine: 190–450 $\mu\text{g}/24\text{ h}$). The only significant difference found during the test was for noradrenalin of the third urine (day 25) between P and T groups with a larger mean value for the T group ($P = 0.0045$).

Discussion

The purpose of this study was to evaluate a potential effect of multiple AAS oral administrations on running performances and on the physical stress level. Up to now, many studies investigated the influence of various AAS on muscular performance and recovery after strength and/or resistance exercise (Bhasin et al. 1996; Kadi 2000; Kuipers et al. 1993). To our knowledge, the endurance factor has never been studied in parallel with a chronic oral intake of testosterone and/or

nandrolone or its precursors. These two products were chosen because they seem to be the AAS most used in sports (WADA 2005). Moreover, oral treatments require an ester of testosterone (Wilson and Griffin 1980) because of the rapid hepatic metabolism of pure testosterone. Norandrostenedione is a recurrent contamination compound of nutritional supplements that are more and more used by athletes. For these reasons, the type of AAS and the doses used in our study could be considered as a good mimical representation of what might be used in professional and amateur sports. Nevertheless, the results of this study may not be directly transferred to all other AAS which could lead to more significant effects.

Exercise tests

To be able to assess a possible beneficial effect of AAS, the training protocol was specifically designed for endurance training based on training regimes used in the preparation of elite athletes and aimed at reaching a high level of physical and mental exhaustion at the

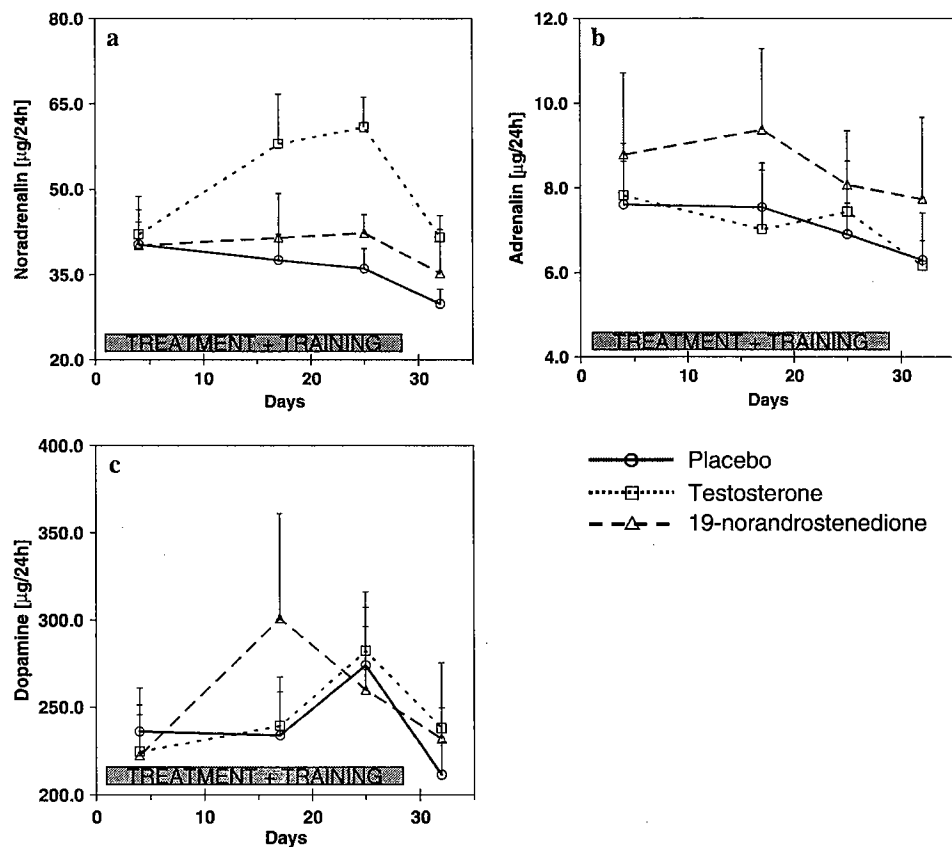


Fig. 5 Mean values (\pm SEM) of urinary excreted noradrenalin (a), adrenalin (b) and dopamine (c) for the placebo (open circles, unbroken line), testosterone (open square, dotted line) and

19-norandrostenedione (open triangle, broken line) groups. The training and treatment period is also depicted

end of the 4 weeks training in the volunteers (Fig. 1). This aim was well achieved according to the oral feedback obtained from the subjects.

For exercise testing, we used a protocol that has been used and validated for the evaluation of running performance in previous studies (Roeker et al. 1998). There are many physiological descriptors for the evaluation of performance. We choose the relation between running speed, serum lactate concentration and heart rate for the description of effort. From these parameters, a reliable prediction of race times in running competitions is possible (Roeker et al. 1998) and thus this test setting has a high practical impact for high performance sport. These three parameters were evaluated at two different time points during the treadmill tests. First, at the IAT and secondly at exhaustion (max) of the subjects. At the beginning of the protocol, only two values were found to be significantly different between N and T groups. T group subjects had a lactate concentration slightly under the one of the N group at the IAT and a maximal speed higher than N group individuals. These points toward the fact that the initial physical

level of the 25 individuals was not uniform and is a recurrent problem in the clinical studies made on human volunteers (Hartgens and Kuipers 2004) that is very difficult to avoid because of the high number of subjects that are required for such studies. Even if these differences exist, in the present study, there are quite negligible ($P = 0.02$ and 0.03). The P, T and N groups were therefore considered as comparable with each other. The variables measured during the treadmill tests performed after the training program (day 38) showed no variation between the three groups ($P > 0.05$) which can be interpreted as an identical intergroup response to training, regardless of the administered substances.

The intragroup performance variation following the training month was assessed in calculating the difference of the same values described above measured during the post and the pre-treatment treadmill tests (Fig. 2). Once again no clear conclusions can be drawn even if $\Delta_{\text{speed IAT}}$ of the N group subjects and $\Delta_{\text{lactate IAT}}$ for N and P groups significantly increased and decreased, respectively. Only one parameter ($\Delta_{\text{max speed}}$)

increased considerably for the three groups. It can be speculated that this increase of the maximal speed might be due to a uniform effect of the training program in the three groups.

On the other hand, there is an adaptation phenomenon of the subjects to the treadmill which implies that an individual will never perform two identical exercise tests. In addition, the intraindividual variation of the performance test might be greater than a potential effect of training or treatment. This is a recurrent problem in sports medicine (Hopkins 2004) and training planning. Therefore the differences observed between the performance related variables at the beginning and the end of the study are not important enough to prove that the investigated oral AAS are useful to increase endurance performance. It has to be noticed that the number of subjects in our study might be insufficient to unmask small effects of AAS. Indeed, it is possible that the performance is influenced by the oral intake of AAS in a range of 2–5%. Such a difference, e.g. in the speed at IAT might have a significant impact in a top level sporting competition but would require higher volunteer number to be revealed.

Hormone profiles

Many authors have investigated the effects of exercise on the endocrine system. While some studies demonstrated that physical efforts do not influence the concentrations of serum hormones like testosterone (Smilios et al. 2003), others clearly showed that there is a hormone variation (testosterone, LH and cortisol) due to exercise (Bonen et al. 1979; Galbo et al. 1977; Kuoppasalmi et al. 1980). These contradictory results could be explained by a reaction dependent on several factors like intensity, duration and mode of exercise and also by the training level of the subjects (Tremblay et al. 2004, 2005; Urhausen et al. 1995; Viru 1992). For this reason, the study protocol is a key criterion to consider before comparing the results reported in the literature. It is well documented that during and just after an endurance effort, testosterone (anabolic hormone) falls under the basal values whereas cortisol (catabolic hormone) concentration increases, which implies a decrease of the anabolic/catabolic (T/cortisol) ratio. It has been suggested that such adaptation allow amino acids to be redirected from protein synthesis to gluconeogenesis needed in stress situation (Duclos et al. 1996; Nindl et al. 2001). Up to now, only the immediate evolution (hours after efforts) of these biological markers has been investigated. With this study we focused on the middle and long-term changes of the hormonal profiles. The hormonal responses depicted in Fig. 3 show no significant

variation for total testosterone, cortisol and LH in the three groups. According to previously reported results (Fernandez-Garcia et al. 2002; Wheeler et al. 1991) a drop of the testosterone serum concentrations should have been observed but none of the three groups showed such picture. Moreover, the intake of testosterone undecanoate seems not to have any effect on the testosterone concentrations meaning that the oral doses were probably not sufficient enough to influence the hypothalamic-pituitary-testicular (HPT) axis. At the end of the training program, the volunteers could be considered as endurance trained athletes. Thus the final decline of testosterone, for the three groups, could be explained by a down regulation of the HPT axis that was already observed in this kind of athletes (Hackney et al. 1990, 1998). Free and bioactive testosterone concentrations were also calculated through the measurement of SHBG (data not shown). As this latter did not vary significantly, the free and bioactive testosterone profiles are the same as the one of total testosterone.

Cortisol has been proven to be one of the essential adaptation hormone during exercise (Viru and Viru 2004) but the variations are dependent of the intensity and duration of effort (Viru et al. 1992). As testosterone, cortisol serum concentration is quite stable during the treatment phase and drops significantly 10 days after the end of the training. The fact that cortisol levels did not change during the training was already observed by Keizer et al. (1989) and this might be explained by the rapid return of this hormone to the basal value. The final decrease of cortisol is probably an indicator of a complete recovery of the volunteers in whom the catabolic activity is not present any more.

Intuitively, longitudinal follow up of athletes during 1 month of endurance training should have resulted in a testosterone decline and then by a retro control feed back to a stimulation of the LH production (Matsumoto and Bremner 1984). As testosterone does not vary, LH remains stable during the entire study. These results have already been reported in the literature. MacConnie et al. (1986) stipulated that this non response of LH may be caused by the prolonged, repetitive elevations of gonadal steroids and other hormones known to suppress gonadotropin-releasing hormone secretion that are elicited by daily exercise. The T/LH serum and urinary ratios have been proven as an indicator of high injection dose of testosterone administration (Kicman et al. 1990). The multiple oral doses of testosterone undecanoate taken by the T groups subjects seem not be sufficient to have a significant impact on the HPT axis as testosterone and T/LH ratio are rather constant and not different from the two other groups, along the study.

All these results could indicate that the HPT axis has not been influenced by the duration (1 month) and intensity of the efforts. As the subjects trained outdoor, the conditions were not controlled as well as in laboratory settings. Nevertheless, the volunteers had to report their daily exercise (time of run and intensities) in order to allow a control of the protocol by the investigators. Except this control, the diet and others physical activities were not monitored. This is probably not an explanation of the relative hormonal stability which is most likely explained by the times of the blood withdrawals (between 6.30 and 8 am, 48 h after the previous medication).

Muscular activity biomarkers

To have an indirect measurement of the physical stress, the global time course of CK, ASAT and urea was assessed during the training month (Fig. 4). CK is an enzyme important for the muscular contraction cycle and present in striated and cardiac muscles. An augmentation of this biomarker could result from a release of the damaged muscles due to intense physical effort (Ebbeling and Clarkson 1989). The basal values of the three groups are slightly above the reference range values. This observation is common in people practicing sports on a regular base. Even if no statistical changes were observed for CK, the profile indicated that at the end of the two first training weeks (days 5 and 12) the subjects have highly solicited their muscles. On days 19 and 26, relatively low CK serum levels could express the organism adaptation to the training stress. ASAT is also contained in striated muscles and increase as well as CK after strenuous bout of exercise. The profile of ASAT is comparable with the one of the CK and confirms the observations made above. Urea is the final product of the amino acids degradation. The dosage of serum urea is mainly performed to evaluate the renal function which can be altered by intense physical exercise (Poortmans 1995). Once again, for testosterone group, the profile illustrates the exercise intensity but for N and P groups no clear pattern was detected. In the case of a better physical stress tolerance following the intake of oral AAS, it can be expected that one or more of these three biomarkers that were chosen for an indirect measurement of stress, would be more constant during an intense training program. Our results do not show any intergroup difference in the serum CK, ASAT or urea profiles. Subsequently, no beneficial effects on physical stress could be attributed to oral testosterone undecanoate or norandrostenedione.

Inflammatory and stress markers

IL-6 has been proven to be an inflammation responsive cytokine that drastically increase after strenuous exercise (Pedersen et al. 1998) and also after prolonged running (Ostrowski et al. 1999). Some authors postulated that IL-6 could be involved in hormonal response to training (Steensberg et al. 2003; Steinacker et al. 2004). The main part of IL-6 analysis performed on the serum samples were below the detection limit of the immunological test used and no statistical interpretation was possible. According to these results, we are not able to confirm an increase of IL-6 for both groups and this parameter seems therefore not useful for a long-term stress evaluation.

To have an indirect feed back of the physiological stress generated by the hard training, catecholamines were quantified in 24 h collected urines (Fig. 5). These are hormones secreted in stress situations. Changes in resting plasma and/or urinary catecholamines (adrenalin and noradrenalin) have been suggested as possible tools for monitoring the impact of training load and/or overload (Lehmann et al. 1997; Mackinnon et al. 1997). It has also been shown that excretion of catecholamines is correlated with training load (Atlaoui et al. 2006) and emotional stress (Lehmann et al. 1988). Between the three measured catecholamines, only noradrenalin seems to be influenced by effort as an increase is observed for the T group. Even if this is a significant change, no reasonable explanation was found.

Limitations

Some limitations must be taken into consideration. First, the fact that training and treatment are done at the same time complicated the interpretation of the results. Second, the statistical models used are of limited significance for a small number of volunteers. More subjects could have unmasked differences in a range of 2–5%. However, small differences would be of great importance for professional athletes that compete at the highest level. Our observations are not in accordance with the major studies made in the field of physical stress and hormone response following endurance exercise, probably because contrasting most other studies, we tried to investigate the development of hormones and other physiological markers in a time period of days rather than hours. The authors estimate that the training intensity and volume were sufficient to trigger typical hormonal responses. Third, the doses of AAS administered in our investigation could have been too low to observe any performance or recovery improvements. However,

our doses were supposed to mimic the ones taken by athletes and higher amounts would not be realistic in the antidoping field. Maybe that a prolonged medication period could lead to statistical differences regarding the investigated physiological parameters between AAS groups and placebo. The effect of AAS could also be psychological and, in that case might not be measurable through physiological parameters.

Conclusion

This study investigated the effect of multiple oral AAS intake on performance capacity and markers of physical stress of endurance trained athletes. Based on the results, it is not obvious that a regular exogenous supply of testosterone undecanoate or 19-norandrostenedione (a nandrolone precursor) reduces physical stress or improves endurance performance of the athlete.

According to these observations, further investigations with higher number of subjects and different treatment times are needed to support the hypothesis that AAS have a real impact on the organism to improve recovery during and after endurance efforts.

Acknowledgements We would like to acknowledge the Federal Office of Sports (BASPO) of Switzerland which provided the financial support for this study and also the 30 participants coming mainly from the Institute of Sport Sciences and Physical Education (ISSEP, University of Lausanne). The authors are also grateful towards Mr Rodolphe Gerber from Bühlmann Laboratories AG (Schönenbuch, Switzerland), Polyanalytic SA (Morges, Switzerland) and the department of sport medicine of the clinical University of Freiburg (Germany) for their technical support for the blood and urine sample analysis. The investigators are thankful towards Sigma Sport (Germany) for having lent the heart rate monitors (Fitwatch).

References

- Alway SE, Starkweather LA (1993) Effect of anabolic steroids on new fiber formation and fiber area during stretch-overload. *J Appl Physiol* 74:832–837
- Atlaoui D, Duclos M, Gouarne C, Lacoste L, Barale F, Chatard JC (2006) 24-hr urinary catecholamine excretion, training and performance in elite swimmers. *Int J Sports Med* 27(4):314–321
- Baume N, Avois L, Schweizer C, Cardis C, Dvorak J, Cauderay M, Mangin P, Saugy M (2004) [¹³C] Nandrolone excretion in trained athletes: interindividual variability in metabolism. *Clin Chem* 50:355–364
- Baume N, Saudan C, Desmarchelier A, Strahm E, Sottas PE, Bagutti C, Cauderay M, Schumacher YO, Mangin P, Saugy M (2006) Use of isotope ratio mass spectrometry to detect doping with oral testosterone undecanoate: Inter-individual variability of (13)C/(12)C ratio. *Steroids* (in press)
- Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Tricker R, Shirazi A, Casaburi R (1996) The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med* 335:1–7
- Bonen A, Ling WY, MacIntyre KP, Neil R, McGrail JC, Belcastro AN (1979) Effects of exercise on the serum concentrations of FSH, LH, progesterone, and estradiol. *Eur J Appl Physiol Occup Physiol* 42:15–23
- Boone JB Jr, Lambert CP, Flynn MG, Michaud TJ, Rodriguez-Zayas JA, Andres FF (1990) Resistance exercise effects on plasma cortisol, testosterone and creatine kinase activity in anabolic-androgenic steroid users. *Int J Sports Med* 11:293–297
- Bricout V, Wright F (2004) Update on nandrolone and norsteroids: how endogenous or xenobiotic are these substances? *Eur J Appl Physiol* 92:1–12
- Broeder CE (2003) Oral andro-related prohormone supplementation: do the potential risks outweigh the benefits? *Can J Appl Physiol* 28(1):102–116
- Dickhuth HH, Huonker M, Münzel T, Drexler A, Berg A, Keul J (1991) Individual anaerobic threshold for the evaluation of competitive athletes and patients with left ventricular dysfunction. In: Bachl TG, Löllgen H (eds) *Advances in ergometry*. Springer, Berlin Heidelberg New York, pp 173–179
- Duclos M, Corcuff JB, Rashedi M, Fougere V, Manier G (1996) Does functional alteration of the gonadotropic axis occur in endurance trained athletes during and after exercise? A preliminary study. *Eur J Appl Physiol Occup Physiol* 73:427–433
- Ebbeling CB, Clarkson PM (1989) Exercise-induced muscle damage and adaptation. *Sports Med* 7:207–234
- Fernandez-Garcia B, Lucia A, Hoyos J, Chicharro JL, Rodriguez-Alonso M, Bandres F, Terrados N (2002) The response of sexual and stress hormones of male pro-cyclists during continuous intense competition. *Int J Sports Med* 23:555–560
- Friedl KE, Dettori JR, Hannan CJ Jr, Patience TH, Plymate SR (1991) Comparison of the effects of high dose testosterone and 19-nortestosterone to a replacement dose of testosterone on strength and body composition in normal men. *J Steroid Biochem Mol Biol* 40:607–612
- Galbo H, Hummer L, Peterson IB, Christensen NJ, Bie N (1977) Thyroid and testicular hormone responses to graded and prolonged exercise in man. *Eur J Appl Physiol Occup Physiol* 36:101–106
- Hackney AC, Sinning WE, Bruot BC (1990) Hypothalamic-pituitary-testicular axis function in endurance-trained males. *Int J Sports Med* 11:298–303
- Hackney AC, Fahrner CL, Gullledge TP (1998) Basal reproductive hormonal profiles are altered in endurance trained men. *J Sports Med Phys Fitness* 38:138–141
- Halsen SL, Jeukendrup AE (2004) Does overtraining exist? An analysis of overreaching and overtraining research. *Sports Med* 34(14):967–981
- Hartgens F, Kuipers H (2004) Effects of androgenic-anabolic steroids in athletes. *Sports Med* 34:513–554
- Haupt HA, Rovere GD (1984) Anabolic steroids: a review of the literature. *Am J Sports Med* 12:469–484
- Hopkins W (2004) How to interpret changes in an athletic performance test. *Sportscience* 8:1–7. <http://www.sportsci.org/jour/04/wghtests.htm>. Accessed Sept 25, 2005
- Hug M, Mullis PE, Vogt M, Ventura N, Hoppeler H (2003) Training modalities: over-reaching and over-training in athletes, including a study of the role of hormones. *Best Pract Res Clin Endocrinol Metab* 17:191–209
- Jakob E, Hoffman R, Fuchs F, Stüwe-Schlobies M, Donike M, Keul J (1988) Testosteronapplikation und Leistungsfähigkeit bei Skilangläufern. *Dtsch Z Sport Med* 39:41–45
- Kadi F (2000) Adaptation of human skeletal muscle to training and anabolic steroids. *Acta Physiol Scand* 646(Suppl):1–52

- Keizer H, Janssen GM, Menheere P, Kranenburg G (1989) Changes in basal plasma testosterone, cortisol, and dehydroepiandrosterone sulfate in previously untrained males and females preparing for a marathon. *Int J Sports Med* 10(Suppl 3):S139–S145
- Kenyon AT, Sandiford I, Bryan AM, Knowlton K, Koch FC (1938) The effect of testosterone propionate on nitrogen, electrolytes, water and energy metabolism in eunuchoidism. *Endocrinology* 23:135–144
- Keul J, Deus B, Kindermann W (1976) Anabole hormone: Schädigung, Leistungsfähigkeit und Stoffwechsel. *Med Klin* 71:497–503
- Kicman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee MN, Southan GJ, Wheeler MJ (1990) Criteria to indicate testosterone administration. *Br J Sports Med* 24:253–264
- Kuipers H, Keizer HA (1988) Overtraining in elite athletes. Review and directions for the future. *Sports Med* 6:79–92
- Kuipers H, Peeze Binkhorst FM, Hartgens F, Wijnen JA, Keizer HA (1993) Muscle ultrastructure after strength training with placebo or anabolic steroid. *Can J Appl Physiol* 18:189–196
- Kuoppasalmi K, Naveri H, Harkonen M, Adlercreutz H (1980) Plasma cortisol, androstenedione, testosterone and luteinizing hormone in running exercise of different intensities. *Scand J Clin Lab Invest* 40(5):403–409
- Lehmann M, Jakob E, Roscher E, Tusch R, Keul J (1988) Ski-flying: related catecholamin excretion compared with cross-country skiing. *Int J Sports Med* 9:438–442
- Lehmann M, Foster C, Keul J (1993) Overtraining in endurance athletes: a brief review. *Med Sci Sports Exerc* 25:854–862
- Lehmann MJ, Lormes W, Opitz-Gress A, Steinacker JM, Netzer N, Foster C, Gastmann U (1997) Training and overtraining: an overview and experimental results in endurance sports. *J Sports Med Phys Fitness* 37:7–17
- MacConnie SE, Barkan A, Lampman RM, Schork MA, Beitins IZ (1986) Decreased hypothalamic gonadotropin-releasing hormone secretion in male marathon runners. *N Engl J Med* 315:411–417
- Mackinnon LT, Hooper SL, Jones S, Gordon RD, Bachmann AW (1997) Hormonal, immunological, and hematological responses to intensified training in elite swimmers. *Med Sci Sports Exerc* 29:1637–1645
- Matsumoto AM, Bremner WJ (1984) Modulation of pulsatile gonadotropin secretion by testosterone in man. *J Clin Endocrinol Metab* 58(4):609–614
- Nindl BC, Kraemer WJ, Deaver DR, Peters JL, Marx JO, Heckman JT, Loomis GA (2001) LH secretion and testosterone concentrations are blunted after resistance exercise in men. *J Appl Physiol* 91:1251–1258
- Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK (1999) Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol* 515:287–291
- Pedersen BK, Ostrowski K, Rohde T, Bruunsgaard H (1998) The cytokine response to strenuous exercise. *Can J Physiol Pharmacol* 76:505–511
- Petibois C, Cazorla G, Poortmans JR, Deleris G (2002) Biochemical aspects of overtraining in endurance sports: a review. *Sports Med* 32(13):867–878
- Pongchaiyakul C, Kosulwat V, Rojroongwasinkul N, Charoenkiatkul S, Thepsuthammarat K, Laopaiboon M, Nguyen TV, Rajatanavin R (2005) Prediction of percentage body fat in rural Thai population using simple anthropometric measurements. *Obes Res* 13(4):729–738
- Poortmans JR (1995) Renal response to exercise in healthy and diseased patients. *Nephrologie* 16:317–324
- Röcker K, Schotte O, Niess A, Heitkamp HC, Dickhuth HH (1997) Laufbandtestdaten und Wettkampfprognosen für den Langstreckenlauf. *D Z Sportmed* 48:315–323
- Roecker K, Schotte O, Niess AM, Horstmann T, Dickhuth HH (1998) Predicting competition performance in long-distance running by means of a treadmill test. *Med Sci Sports Exerc* 30:1552–1557
- Roecker K, Niess AM, Horstmann T, Striegel H, Mayer F, Dickhuth HH (2002) Heart rate prescriptions from performance and anthropometrical characteristics. *Med Sci Sports Exerc* 34:881–887
- Rozenek R, Rahe CH, Kohl HH, Marple DN, Wilson GD, Stone MH (1990) Physiological responses to resistance-exercise in athletes self-administering anabolic steroids. *J Sports Med Phys Fitness* 30:354–360
- Smilios I, Piliandis T, Karamouzis M, Tokmakidis SP (2003) Hormonal responses after various resistance exercise protocols. *Med Sci Sports Exerc* 35:644–654
- Steensberg A, Fischer CP, Keller C, Møller K, Pedersen BK (2003) IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am J Physiol Endocrinol Metab* 285:E433–E437
- Steinacker JM, Lormes W, Reissnecker S, Liu Y (2004) New aspects of the hormone and cytokine response to training. *Eur J Appl Physiol* 91:382–391
- Tamaki T, Uchiyama S, Uchiyama Y, Akatsuka A, Roy RR, Edgerton VR (2001) Anabolic steroids increase exercise tolerance. *Am J Physiol Endocrinol Metab* 280:E973–E981
- Tremblay MS, Copeland JL, Van Helder W (2004) Effect of training status and exercise mode on endogenous steroid hormones in men. *J Appl Physiol* 96:531–539
- Tremblay MS, Copeland JL, Van Helder W (2005) Influence of exercise duration on post-exercise steroid hormone responses in trained males. *Eur J Appl Physiol* 94:505–513
- Urhausen A, Stein R, Kindermann W (1989) Metabolismus und hormonelles Verhalten bei Ausdauertrainierten unter Testosteronapplikation. *Dtsch Z Sport Med* 40:312–320
- Urhausen A, Gabriel H, Kindermann W (1995) Blood hormones as markers of training stress and overtraining. *Sports Med* 20:251–276
- Viru A (1992) Plasma hormones and physical exercise. *Int J Sports Med* 13:201–209
- Viru A, Viru M (2004) Cortisol—essential adaptation hormone in exercise. *Int J Sports Med* 25:461–464
- Viru A, Karelson K, Smirnova T (1992) Stability and variability in hormonal responses to prolonged exercise. *Int J Sports Med* 13:230–235
- WADA, The World Anti-Doping Agency (2005) 2004 Adverse analytical findings reported by Accredited Laboratories, May 2005. <http://www.wada-ama.org>
- Wasserman K, Hansen JE, Sue DY, Stringer WW, Whipp BJ (1999) Principles of exercise testing and interpretation, 4th edn. Lippincott, Williams & Wilkins, Baltimore, pp 568
- Wheeler GD, Singh M, Pierce WD, Epling WF, Cumming DC (1991) Endurance training decreases serum testosterone levels in men without change in luteinizing hormone pulsatile release. *J Clin Endocrinol Metab* 72:422–425
- Wilson JD, Griffin JE (1980) The use and misuse of androgens. *Metabolism* 29:1278–1295

Exogenous testosterone, aggression, and mood in eugonadal and hypogonadal men

Daryl B. O'Connor^{a,*}, John Archer^b, W. Morton Hair^a, Frederick C.W. Wu^a

^a*Department of Endocrinology, Manchester Royal Infirmary, Manchester M13 9WL, UK*

^b*Department of Psychology, University of Central Lancashire, Preston, Lancashire PR1 2HE, UK*

Received 19 July 2001; received in revised form 8 October 2001; accepted 15 January 2002

Abstract

To investigate (1) the effects of exogenous testosterone (T) on self- and partner-reported aggression and mood and (2) the role of trait impulsivity in the T–aggression relationship. Thirty eugonadal men with partners were randomized into two treatment groups to receive: (1) 200 mg im T enanthate weekly for 8 weeks or (2) 200 mg im sodium chloride weekly for 8 weeks. Eight hypogonadal men received 200 mg im T enanthate biweekly for 8 weeks. All groups completed a battery of behavior measures at baseline (Week 0) and at Weeks 4 and 8. Cognitive and motor impulsivity were the only predictors of self-reported total aggression (over and above age and T levels) at Weeks 0, 4, and 8. No significant changes in aggression or mood levels were found in the eugonadal-treated group. Significant reductions in negative mood (tension, anger, and fatigue) followed by an increase in vigor were found in response to T treatment in the hypogonadal group. These results demonstrate that inability to control one's behavior when such control is required by a particular situation (impulsivity) was found to significantly predict levels of aggression over and above age and T level. These data do not support the hypothesis that supraphysiological levels of T (within this range) lead to an increase in self- and partner-reported aggression or mood disturbances. Instead, for the first time, this study has identified the high level of negative affect experienced by hypogonadal patients. These findings have implications for T replacement therapy and male contraception. © 2002 Published by Elsevier Science Inc.

Keywords: Testosterone; Aggression; Hostility; Impulsivity; Hypogonadal; Eugonadal

1. Introduction

The effect of testosterone (T) on behavior, and in particular aggression, has received considerable attention over the last decade [1–4]. This has been prompted by several developments. Firstly, the high-profile media coverage of incidents of 'steroid rage' seemingly associated with the abuse by strength athletes of androgenic–anabolic steroids (AAS) [5]. Secondly, the use of exogenous T clinically as part of the development of a reversible, hormonal contraceptive for men [6], its use for replacement therapy in HIV illness [7], and for treating the psychological and physiological effects of aging in men [8].

In general, these studies can be characterised as: (1) Comparisons between violent criminals and nonviolent controls on some measure of aggression [9–11]. These studies

generally demonstrate higher T levels among the more violent men than the less violent ones. (2) Correlational studies investigating the degree of association among endogenous T levels, aggression, and hostile feelings. These studies have shown conflicting results, with stronger associations being found when behavioral and observational measures have been employed compared to trait measures of aggression [9,12,13]. (3) Experimental studies where T levels have been manipulated into the supraphysiological range [1,14–17]. The existing experimental studies have yielded varied results, and they are difficult to interpret owing to differences in experimental manipulations and in the dependent variables.

Here, we examine reports providing measures of direct aggression for baseline or placebo phases, and for phases when levels of T were elevated to supraphysiological levels. We calculated effect sizes (*g*) from the means and standard deviations provided in the papers, for the differences between baseline and T conditions, and for the differences between placebo and T conditions [18]. Su et al. [16]

* Corresponding author. School of Psychology, University of Leeds, Leeds, LS2 9JT, UK. Tel.: +44-113-233-5727; fax: +44-113-233-5749.

E-mail address: daryl@psychology.leeds.ac.uk (D.B. O'Connor).

injected 20 men with placebo, followed by a low dose of methyltestosterone, and then a high-dose methyltestosterone. Their psychological measures were mainly concerned with mood, but included ratings of 'violent feelings' on a visual analogue scale. Comparing baseline with the high-dose phase yielded a value of $g=.28$; comparing baseline with the low-dose phase yielded a value of $g=.09$. However, the authors report trends for an increase in anger and violent feelings in the high-dose group. Yates et al. [17] reported a double-blind study of 31 men involving 100, 250, and 500 mg of T cypionate. For self-ratings on the assault scale of the Buss–Durkee Hostility Inventory (BDHI), values indicated no difference between baseline or placebo and the average of all three doses of T ($g=.08$ for baseline and $g=0$ for placebo). However, values indicating an increase on this scale were found when the ratings were provided by an informant rather than the person himself ($g=.47$ for comparisons with baseline and $g=.17$ for placebo). Pope et al. [15] reported values on a laboratory measure of aggression [the Point Subtraction Aggressive Paradigm (PSAP) by Cherek et al. [19]] for baseline, placebo, and treatment phases for 27 men receiving up to 600 mg T cypionate. The values were $g=.57$ for the baseline comparison and $g=.51$ for the treatment comparison, indicating a rise in aggression following T treatment. Although the Aggression Questionnaire (AQ), the modern version of the BDHI, was used in this study the values presented were aggregates for the four subscales: physical and verbal aggression, hostility, and anger. An initial report based on 8 of the 51 men completing this measure [20] indicated an increase in physical aggression following treatment ($g=.79$), but a much smaller one for verbal aggression ($g=.20$). In the complete sample, the values for the total AQ scores were around zero, indicating no change in this composite measure of direct aggression, hostility, and anger.

Over the three studies, when effect sizes are calculated, there is some evidence for increases in measures of direct aggression following T administration, but this is inconsistent, and nonsignificant in most cases.

Experimental studies have generally not found evidence for a T–anger relationship [1,14,21]. For example, Tricker et al. [1] administered a large supraphysiological dose of T enanthate (600 mg/week) to a sample of healthy men in a double-blind, placebo-controlled fashion and assessed both self-reported and partner-reported mood and anger. Their study failed to detect any effects on angry behavior or mood. More intriguingly, Bjorkqvist et al. [21] found evidence for a placebo effect, where men who received a placebo in a double-blind experiment reported higher scores on self-estimated anger, irritation, impulsivity, and frustration than those receiving T orally (Panteston). These increases were also supported by the observer estimates. However, these findings should be interpreted with caution given that the orally administered T did not increase circulating T levels into the supraphysiological range.

One concern in this area [21] is that positive results are more readily reported than null ones and that investigators are less likely to submit manuscripts that accept null hypotheses. These findings also suggest that the relationship between T (or steroid use) and aggressive behavior may not be a direct, causal one. With this in mind, it is important to note that few experimental studies have investigated the possible role of individual difference variables, such as impulsivity, in their experimental paradigms [3]. Research in this area is also limited by over-reliance on self-report data, with few studies incorporating observations from a partner or significant other (e.g. Ref. [1]). There is also a dearth of controlled investigations of the effects of T administration on aggressive, hostile behavior, and mood in young hypogonadal men who have low levels of T [22].

The aim of this study was to investigate the dose–response relationship among T levels, behavior, and mood in men. A eugonadal-treated group receiving weekly injections of supraphysiological levels of T and a hypogonadal group receiving physiological replacement levels of T were compared with a eugonadal-placebo control group, over an 8-week period. To improve on previous studies, a wide range of measures were used, including self- and partner-reported aggression, anger and hostility, self-esteem, assertiveness, irritability, and moods. Trait impulsivity was also assessed at baseline.

2. Method

2.1. Subjects

Thirty healthy male volunteers (mean age=28.2 years; range 19–45 years) and 8 hypogonadal male patients (mean age=30.8 years; range 23–40 years) participated in the study. The eugonadal men were recruited from local radio and newspaper advertisements. The hypogonadal patients were recruited from the Department of Endocrinology, Manchester Royal Infirmary and Hope Hospital, Manchester, UK. The hypogonadal group consisted of three men with Kallmann's syndrome, two with Klinefelter's syndrome, and three who had a bilateral orchidectomy receiving maintenance T replacement for a mean of 5 years (range 0–14 years).

2.2. Design

Volunteers who met the admission criteria after medical screening were randomised into two treatment groups ($n=15$) to receive (1) in the eugonadal-treated group: 200 mg im T enanthate (Cambridge Laboratories, UK) once weekly for 8 weeks or (2) in the eugonadal-placebo group: 200 mg im 0.9% sodium chloride solution weekly for 8 weeks. The eugonadal-treated group received 200 mg T, weekly for two reasons: (a) the pharmacokinetics of this

Table 1

Correlations among the independent variables (age, T, and impulsivity) and total aggression scores for all groups (All), eugonadal group (Eug: treated and placebo), and the hypogonadal group (hypo)

| Variables | Week 0 | | | Week 4 | | | Week 8 | | |
|-------------------------|--------|-------|------|--------|-------|------|--------|-------|------|
| | All | Eug | Hypo | All | Eug | Hypo | All | Eug | Hypo |
| Age | -.04 | -.16 | .00 | .10 | .01 | -.01 | -.08 | -.22 | -.10 |
| T | -.41* | -.21 | .48 | -.12 | .15 | .09 | -.15 | .02 | -.33 |
| Nonplanning impulsivity | .05 | .18 | .57 | -.15 | .00 | .49 | .02 | .21 | .65 |
| Cognitive impulsivity | .60** | .63** | .39 | .53** | .57** | .30 | .65** | .73** | .22 |
| Motor impulsivity | .56** | .48* | .56 | .56** | .54** | .30 | .63** | .56** | .59 |

* $P < .05$.

** $P < .01$.

T preparation are well-documented [23] and (b) this dosage has been used successfully in male contraceptive clinical trials [23]. One participant from the eugonadal-treated group had to withdraw from the study before Week 4 for personal reasons unrelated to the study. A wash-out period of 6–8 weeks was required for the hypogonadal group (to allow endogenous T levels to fall to the low hypogonadal range < 6 nmol/l, the normal range being 10–35 nmol/l) before they were admitted to the study. The hypogonadal group received 200 mg im T enanthate biweekly for 8 weeks. No significant age differences were found between the groups [$F(2,35) = 0.97$, ns]. All participants provided written consent. The study was approved by the Central Manchester Research Ethics Committee for Medical Research.

Volunteers completed a battery of validated behavioral scales (see below) at baseline (Week 0) and at Weeks 4 and 8. Mood was assessed weekly. The volunteers completed all the behavioral measures in the privacy of a clinical research room. Blood sampling was performed before receiving intramuscular injection at Weeks 0, 4, and 8. Time of venepuncture was random among the groups, although, generally each participant provided their sample at approximately the same time of day at each of the time points. All plasma samples were stored at -20°C until assay. T was measured using a time-resolved fluoroimmunoassay (AutoDELFA Testosterone Kit) with an assay sensitivity of 0.4 nmol/l.

2.3. Instruments

The Barratt Impulsivity Scale-11 (BIS-11) [24] was administered at baseline to assess trait impulsivity. There are three subscales: nonplanning impulsivity, cognitive impulsivity, and motor impulsivity.

The AQ [25] was administered to assess aggression levels. There are four subscales: physical aggression, verbal aggression, anger, and hostility. A total aggression score was also calculated by summing the four subscales (and is the main dependent variable in Tables 1 and 2). For the purpose of this prospective study, the AQ, which is general in its referents, and can be characterised as a trait measure, was modified slightly to refer to a specific period in the

recent past (i.e. the last 4 weeks). This made the measure more suitable for measuring within-subject variation.

The Partner Aggression Questionnaire (AQ-P) is an adapted version of the AQ [25,26] where the male respondent's partner is asked to rate their partner in relation to each of the AQ items. Since the hypogonadal patients were generally not involved in long-term sexual relationships, no partner data were collected.

The Aggressive Provocation Questionnaire (APQ) [26] is a measure of aggressive responding. It consists of 21 vignettes that are representations of real provocative situations. The respondent is asked to imagine at this moment: (1) how he would feel in each situation (angry, frustrated, and irritated), measured on a five-point Likert scale and, (2) how he would react to each situation by choosing one of five action alternatives categorised as follows: (1) avoid, (2) no response, (3) anger, (4) assertive behavior, and (5) direct aggression. In the present study, analysis concentrated on the total number of aggressive and assertive options chosen across the 21 vignettes.

The State Self-Esteem Scale [27] consists of 20 items that are concerned with performance, social, and appearance aspects of self-esteem.

The Rathus Assertiveness Schedule [28] consists of 30 items, which are concerned with assessing to what extent the participant is assertive in a range of situations. Again for the purpose of this prospective study, the respondents were

Table 2

Stepwise multiple regression analysis: predicting total aggression scores

| Variable | Predictors | <i>t</i> | β | R^2 | <i>P</i> |
|---------------------------|--------------------------|----------|---------|-------|----------|
| Total aggression (Week 0) | 1. Cognitive impulsivity | 4.11 | .63 | .39 | $< .01$ |
| Total aggression (Week 4) | 1. Cognitive impulsivity | 3.53 | .57 | .32 | $< .01$ |
| | 1. Cognitive impulsivity | 2.44 | .41 | | $< .05$ |
| | 2. Motor impulsivity | 2.09 | .35 | .43 | $< .05$ |
| Change in $R^2 = .11$ | | | | | |
| Total aggression (Week 8) | 1. Cognitive impulsivity | 5.51 | .73 | .54 | $< .01$ |
| | 1. Cognitive impulsivity | 4.32 | .60 | | $< .01$ |
| | 2. Motor impulsivity | 2.11 | .29 | .61 | $< .05$ |
| Change in $R^2 = .07$ | | | | | |

instructed to rate themselves over a specific period in the recent past (i.e. last 4 weeks).

The irritability subscale from the BDHI [29] was adapted to be completed along a five-point scale and respondents were instructed to rate themselves over a specific period in the recent past (i.e. last 4 weeks).

The Profile of Mood States (POMS) [30] consists of 65 adjectives that describe feelings and mood. There are six subscales: tension–anxiety, depression–dejection, anger–hostility, vigor–activity, fatigue–inertia, and confusion–bewilderment. A total mood disturbance score was also calculated by subtracting the vigor–activity subscale score from the total summation of all other scales. The analysis concentrated on the mean score for each 2-week period (e.g. mean for Weeks 1 and 2).

2.4. Data analysis

Descriptive statistics were calculated for each of the variables. Preliminary Pearson's Product–Moment Correlational analysis and stepwise multiple regression were used to investigate the predictive relationship between the independent variables (e.g. T level, age, cognitive, motor, and nonplanning impulsivity scores) and the main dependent variable, total aggression (total aggression score includes the summation of each of the scored subscales from the AQ) at each time point. Two-factor analysis of variance for a mixed design was used to assess performance differences across testing sessions (time factor: Weeks 0, 4, and 8) and between groups (group factor: eugonadal-treated, eugonadal-placebo, and hypogonadal groups) for all dependent variables. Multiple comparisons were also made using Scheffe post hoc tests. All data were analysed using SPSS for Windows Version 9.0.

3. Results

3.1. Testosterone levels

T levels are shown in Fig. 1. Two-factor ANOVA revealed significant main effects for time [$F(2,68)=52.96$, $P<.01$] and group [$F(2,34)=38.99$, $P<.01$], and a significant Time \times Group interaction [$F(4,68)=16.39$, $P<.01$]. Post hoc analyses showed that exogenous T significantly increased T levels from a baseline level of 21.7 ± 1.6 – 34.4 ± 1.7 nmol/l at Week 4 to 38.4 ± 2.1 nmol/l at Week 8 in the eugonadal-treated group. In contrast, T levels of the eugonadal-placebo group did not change from a baseline level of 20.1 ± 1.4 nmol/l (Weeks 4 and 8 = 18.9 ± 1.3 and 20.0 ± 1.8 nmol/l, respectively). The hypogonadal group responded to T treatment with a significant increase into the normal range from a baseline level of 3.8 ± 1.1 – 18.1 ± 1.6 nmol/l at Week 4 to 22.4 ± 2.3 nmol/l at Week 8.

3.2. Trait impulsivity

One-way ANOVA revealed a significant difference between the groups for nonplanning impulsivity [$F(2,35)=2.66$, $P<.05$]. The hypogonadal group reported significantly lower levels of nonplanning impulsivity than the eugonadal-placebo group. Neither cognitive [$F(2,35)=0.89$, ns] or motor [$F(2,35)=1.95$, ns] impulsivity showed significant differences.

3.3. Preliminary correlational analysis

Table 1 shows the correlations between the independent variables (age, T level, and impulsivity) and the main dependent variable, total aggression at Weeks 0, 4, and 8.

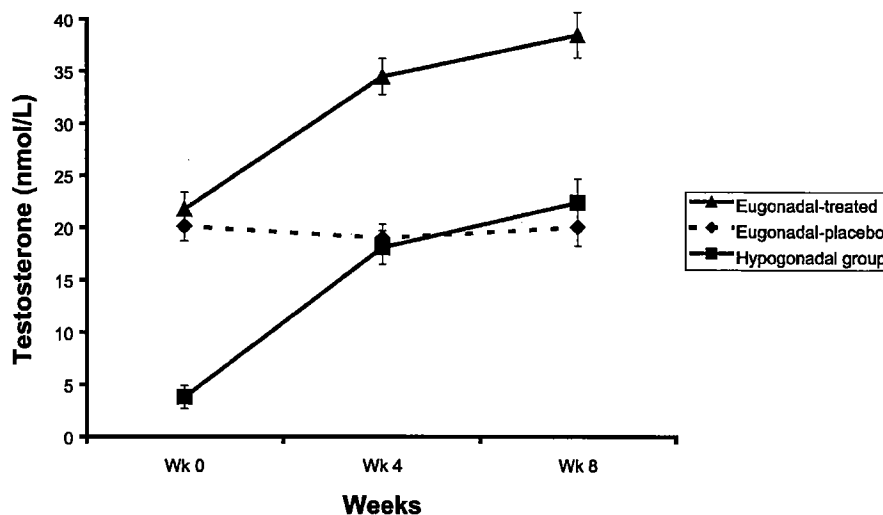


Fig. 1. Testosterone levels in all groups at baseline (week 0), week 4 and week 8.

All groups were first entered into the analysis, to provide greater variability in T levels. The analysis was rerun excluding the hypogonadal group.

At Week 0, only three significant correlations were found. Total aggression was negatively associated with T levels ($r = -.41$, $P < .05$) and positively associated with cognitive and motor impulsivity ($r = .60$, $P < .01$; $r = .56$, $P < .01$; respectively). When the hypogonadal group was excluded, only cognitive and motor impulsivity remained significantly correlated with total aggression levels ($r = .63$, $P < .01$; $r = .48$, $P < .05$; respectively). This indicates that higher levels of trait impulsivity are associated with higher levels of self-reported aggression in the eugonadal men.

At Week 4, two significant correlations were found for all participants combined. Again higher cognitive and motor impulsivity were significantly associated with higher levels of self-reported aggression ($r = .53$, $P < .01$; $r = .56$, $P < .01$; respectively). The correlations were similar after excluding the hypogonadal ($r = .57$, $P < .01$; $r = .54$, $P < .05$; respectively). At Week 8, cognitive and motor impulsivity were the only variables to be significantly associated with total aggression in the combined group and with the hypogonadal men omitted (Table 1).

3.4. Stepwise multiple regression analysis

Stepwise regression was used to further investigate the relationship between impulsivity and total aggression. Table 2 shows the results. At Week 0, only cognitive impulsivity emerged from the analysis, explaining 39% of the variance of total aggression scores. Cognitive impulsivity explained 32% of the variance in total aggression at Week 4, with motor impulsivity explaining an additional

11%. Similarly, at Week 8, cognitive impulsivity explained 54% of the variance in total aggression scores with motor impulsivity explaining a further 7%.

3.5. Aggression Questionnaire

Table 3 summarises self- and partner-reported scores on the AQ for each of the groups.

3.5.1. Physical aggression

No significant main effect was found for time [$F(2,68) = 0.61$, ns] or group [$F(2,34) = 1.29$, ns], suggesting that supra-physiological levels of T did not increase physical aggression. There was a slight trend towards a significant Time \times Group interaction [$F(4,68) = 2.05$, $P = .097$], which is likely to be accounted for by the increase in self-reported physical aggression in the hypogonadal group at Week 4. These findings were further endorsed by the partner reports in the eugonadal-treated and eugonadal-placebo groups. No significant main effects were found for time [$F(2,50) = 2.91$, ns], group [$F(1,25) = 0.80$, ns], or the Time \times Group interaction [$F(2,50) = 0.02$, ns].

3.5.2. Verbal aggression

A significant main effect was found for group [$F(2,34) = 3.39$, $P < .05$]. The hypogonadal group reported significantly higher levels of verbal aggression than the eugonadal-placebo group ($P < .05$). Neither main effect for time [$F(2,68) = 1.47$, ns] or the Time \times Group interaction [$F(4,68) = 0.49$, ns] were significant. Again, no significant main effects were found for partner reports, for time [$F(2,50) = 1.88$, ns], group [$F(1,25) = 0.65$, ns], or the Time \times Group interaction [$F(2,50) = 0.65$, ns].

Table 3

Descriptive statistics for self- and partner-reported scores on the aggression questionnaire at Weeks 0, 4, and 8

| Group | Self report | | | Partner report | | |
|----------------------------|--------------|--------------|--------------|----------------|--------------|--------------|
| | Week 0 | Week 4 | Week 8 | Week 0 | Week 4 | Week 8 |
| <i>Physical aggression</i> | | | | | | |
| Eugonadal-treated | 18.36 (1.61) | 19.07 (1.38) | 18.64 (1.77) | 16.29 (1.68) | 19.07 (1.38) | 17.00 (1.68) |
| Eugonadal-placebo | 18.20 (1.39) | 17.13 (1.22) | 18.06 (1.15) | 15.47 (1.13) | 17.13 (1.22) | 15.71 (1.32) |
| Hypogonadal | 19.75 (1.26) | 22.38 (2.03) | 19.75 (1.43) | | | |
| <i>Verbal aggression</i> | | | | | | |
| Eugonadal-treated | 14.07 (0.69) | 14.14 (0.60) | 13.79 (0.72) | 12.64 (1.10) | 12.23 (1.02) | 12.85 (1.05) |
| Eugonadal-placebo | 13.00 (0.74) | 13.66 (0.65) | 12.40 (0.56) | 11.27 (0.97) | 10.43 (1.05) | 10.64 (0.98) |
| Hypogonadal | 16.00 (1.53) | 16.38 (1.49) | 16.13 (1.69) | | | |
| <i>Hostility</i> | | | | | | |
| Eugonadal-treated | 17.11 (1.47) | 16.29 (1.23) | 15.21 (1.23) | 12.79 (1.09) | 13.69 (1.33) | 14.53 (1.34) |
| Eugonadal-placebo | 16.93 (1.08) | 16.00 (1.48) | 15.26 (1.10) | 12.53 (1.02) | 11.64 (0.80) | 11.21 (0.91) |
| Hypogonadal | 25.25 (2.18) | 26.88 (2.61) | 23.43 (2.38) | | | |
| <i>Anger</i> | | | | | | |
| Eugonadal-treated | 16.43 (1.26) | 16.14 (1.21) | 17.50 (0.74) | 14.57 (1.80) | 14.07 (1.72) | 16.46 (1.40) |
| Eugonadal-placebo | 14.93 (1.04) | 14.60 (1.13) | 15.13 (0.67) | 13.33 (1.38) | 12.79 (1.43) | 14.36 (1.12) |
| Hypogonadal | 20.00 (1.91) | 19.38 (1.38) | 19.50 (1.67) | | | |

Standard error of means are given in parentheses.

3.5.3. Hostility

A significant main effect was found for time [$F(2,68) = 4.89$, $P < .01$] and group [$F(2,34) = 11.11$, $P < .01$]. There was no significant Time \times Group interaction [$F(4,68) = 1.31$, ns]. The hypogonadal group reported significantly higher hostility scores than both the eugonadal-placebo and eugonadal-treated groups ($P < .01$). The significant main effect for time is accounted for by the reduced hostility scores in all groups by Weeks 4 and 8 (Week 4: 18.46 vs. Week 8: 17.27, $P < .05$; Week 0: 19.03 vs. Week 8: 17.27, $P < .01$). The partners generally reported lower levels of hostility than their counterparts. However, there were no significant main effects for time [$F(2,50) = 0.04$, ns], group [$F(1,25) = 2.07$, ns], or the Time \times Group interaction [$F(2,50) = 2.13$, ns].

3.5.4. Anger

No significant main effect was found for time [$F(2,68) = 1.96$, ns] or for the Time \times Group interaction [$F(4,68) = 0.83$, ns]. However, a significant main effect was found for group [$F(2,34) = 4.16$, $P < .05$]. The hypogonadal group reported significantly greater levels of anger compared to the eugonadal-placebo group ($P < .05$). For the partners' scores, a significant main effect was found for time [$F(2,50) = 4.69$, $P < .05$] but not for group [$F(1,25) = 2.07$, ns] or for the Time \times Group interaction [$F(2,50) = 0.20$, ns]. A significant increase in partner-reported anger scores from Weeks 4 to 8 accounted for this significant main effect ($t = 3.47$, $P < .01$).

3.6. Aggressive Provocation Questionnaire

3.6.1. Aggressive actions

No significant main effects were found for either time [$F(2,68) = 1.74$, ns] or group [$F(2,34) = 1.21$, ns]. There was a significant Time \times Group interaction [$F(4,68) = 2.70$, $P = .049$], but post hoc comparisons found no significant differences between any of the cells. These findings provide further support for an absence of a T treatment effect on levels of aggression (Table 4).

3.6.2. Assertive actions

There was a significant main effect for time [$F(2,68) = 4.02$, $P < .05$] but no significant effect for group [$F(2,34) = 0.92$, ns] or for the interaction [$F(4,68) = 0.98$, ns]. The main effect for time was explained by a significant increase in assertive actions from Weeks 0 to 4 (11.95 vs. 13.16; $t = 2.71$, $P < .01$). This trend was sustained at Week 8, although it was nonsignificant ($P = .07$), suggesting that the time effect may be accounted for by an overall placebo effect (Table 4).

3.6.3. Irritability

A significant main effect was found for group [$F(2,34) = 10.04$, $P < .01$], but not for time [$F(2,68) = 2.07$, ns] or for the Time \times Group interaction [$F(4,68) = 0.29$, ns]. Both the

Table 4

Descriptive statistics for behavior measures (on APQ) at Weeks 0, 4, and 8

| Group | Week 0 | Week 4 | Week 8 |
|---------------------------|--------------|--------------|--------------|
| <i>Aggressive actions</i> | | | |
| Eugonadal-treated | 0.93 (0.32) | 1.5 (0.54) | 0.86 (0.34) |
| Eugonadal-placebo | 0.67 (0.33) | 0.40 (0.19) | 1.00 (0.35) |
| Hypogonadal | 1.13 (0.61) | 1.88 (0.67) | 1.62 (0.46) |
| <i>Assertive actions</i> | | | |
| Eugonadal-treated | 11.86 (0.71) | 12.21 (0.80) | 12.71 (0.99) |
| Eugonadal-placebo | 11.47 (0.96) | 13.27 (1.30) | 12.07 (1.11) |
| Hypogonadal | 13.00 (1.12) | 14.63 (1.46) | 14.75 (1.18) |
| <i>Irritability</i> | | | |
| Eugonadal-treated | 28.29 (1.56) | 26.50 (1.51) | 25.64 (1.79) |
| Eugonadal-placebo | 23.87 (1.14) | 24.13 (1.40) | 23.00 (1.28) |
| Hypogonadal | 33.75 (2.40) | 33.75 (1.87) | 32.62 (1.76) |
| <i>State self-esteem</i> | | | |
| Eugonadal-treated | 51.21 (1.54) | 50.29 (1.88) | 51.29 (2.26) |
| Eugonadal-placebo | 48.60 (1.52) | 47.67 (1.32) | 48.53 (1.70) |
| Hypogonadal | 53.00 (2.57) | 53.25 (2.06) | 58.37 (1.88) |
| <i>Assertiveness</i> | | | |
| Eugonadal-treated | 10.21 (2.99) | 19.50 (5.40) | 19.29 (5.13) |
| Eugonadal-placebo | 15.67 (4.31) | 16.73 (4.60) | 19.27 (4.61) |
| Hypogonadal | 4.25 (5.79) | 8.87 (6.04) | 9.00 (5.07) |

Standard error of means are given in parentheses.

eugonadal-treated and eugonadal-placebo groups reported significantly lower levels of irritability overall than the hypogonadal group (Table 4).

3.6.4. State self-esteem

A significant main effect was found for both time [$F(2,68) = 4.86$, $P < .05$] and group [$F(2,34) = 3.28$, $P < .05$], with the Time \times Group interaction approaching significance [$F(4,68) = 2.39$, $P = .059$]. The significant main effect for time is accounted for by the increase in self-esteem scores from 49.86 at Week 4 to 51.70 at Week 8 ($t = 2.13$, $P < .05$), in particular by the increase reported by the hypogonadal group from Weeks 4 to 8 (53.25 vs. 58.37). This finding (Table 4) reflects the trend towards a significant interaction, suggesting the existence of a positive treatment effect on self-esteem in the hypogonadal group.

3.6.5. Assertiveness

A significant main effect was found for time [$F(2,68) = 6.44$, $P < .01$] but not for group [$F(4,68) = 1.24$, ns] or their interaction [$F(2,34) = 1.09$, ns]. Table 4 shows substantial increases in assertiveness at Week 4 compared with Week 0, particularly in the eugonadal-treated and hypogonadal groups.

3.7. Profiles of Mood States

3.7.1. Total mood disturbance

Significant main effects were found for time [$F(4,136) = 6.72$, $P < .01$], for group [$F(2,34) = 13.04$, $P < .01$], and for

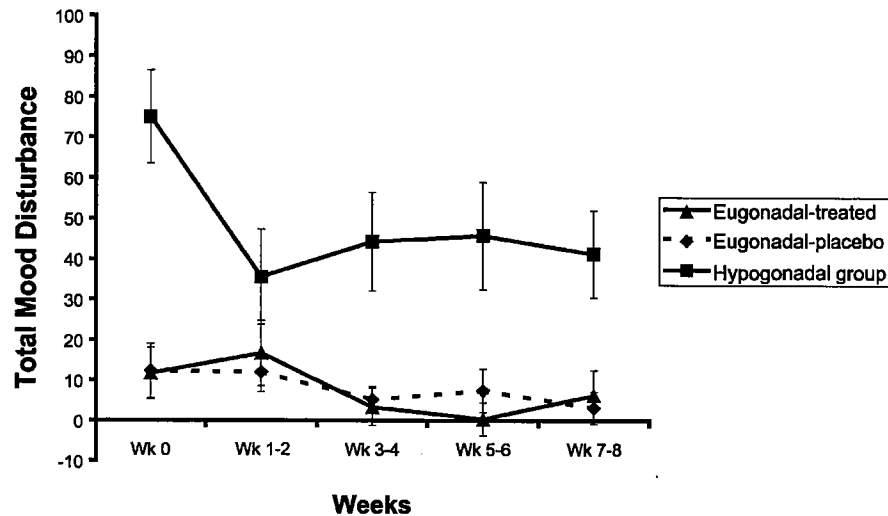


Fig. 2. Total mood disturbance in all groups at baseline (week 0), week 4 and week 8.

the Time \times Group interaction [$F(8,136) = 3.320$, $P < .01$]. Multiple comparisons found that the significant group effect was accounted for by significantly higher levels of total mood disturbance in the hypogonadal group than the eugonadal-treated and eugonadal-placebo groups (see Fig. 2). However, there was a significant reduction in total mood

scores in the hypogonadal group by Weeks 1–2 explaining the significant interaction effect ($t = 3.07$, $P < .05$).

3.7.2. POMS subscales

POMS subscale scores are shown in Table 5. Significant main effects were found for time for tension–anxiety

Table 5
Descriptive statistics for subscales on POMS at Weeks 0, 1–2, 3–4, 5–6, and 7–8

| Group | Week 0 | Week 1–2 | Week 3–4 | Week 5–6 | Week 7–8 |
|-------------------------------|--------------|--------------|--------------|--------------|--------------|
| <i>Tension–anxiety</i> | | | | | |
| Eugonadal-treated | 6.64 (1.09) | 7.71 (1.45) | 5.71 (0.99) | 5.21 (1.10) | 6.68 (1.35) |
| Eugonadal-placebo | 6.40 (1.16) | 5.80 (0.98) | 4.90 (0.76) | 5.30 (0.89) | 4.77 (0.79) |
| Hypogonadal | 17.37 (1.84) | 9.75 (1.12) | 11.63 (1.72) | 11.38 (1.89) | 12.00 (2.01) |
| <i>Depression–dejection</i> | | | | | |
| Eugonadal-treated | 5.07 (1.29) | 5.93 (1.45) | 2.93 (1.20) | 1.68 (0.50) | 1.82 (0.65) |
| Eugonadal-placebo | 5.73 (2.05) | 4.37 (1.34) | 2.70 (0.85) | 3.60 (1.67) | 2.23 (1.20) |
| Hypogonadal | 20.13 (3.83) | 11.25 (3.55) | 12.06 (4.03) | 12.69 (4.08) | 11.44 (3.32) |
| <i>Anger–hostility</i> | | | | | |
| Eugonadal-treated | 7.29 (1.88) | 9.11 (2.20) | 4.86 (1.28) | 3.46 (0.83) | 5.54 (1.79) |
| Eugonadal-placebo | 3.73 (1.33) | 4.03 (0.82) | 4.10 (0.82) | 4.00 (0.99) | 3.13 (0.67) |
| Hypogonadal | 17.75 (2.37) | 12.06 (2.90) | 17.69 (2.70) | 16.88 (2.85) | 14.75 (1.95) |
| <i>Vigor–activity</i> | | | | | |
| Eugonadal-treated | 20.79 (1.63) | 18.36 (1.70) | 18.43 (1.71) | 17.89 (1.87) | 17.79 (2.00) |
| Eugonadal-placebo | 17.06 (1.41) | 16.50 (1.46) | 17.87 (1.24) | 16.47 (0.98) | 16.87 (1.04) |
| Hypogonadal | 10.63 (2.26) | 15.88 (2.22) | 17.06 (1.80) | 14.75 (1.71) | 17.50 (1.75) |
| <i>Fatigue–inertia</i> | | | | | |
| Eugonadal-treated | 7.36 (1.44) | 7.43 (1.25) | 4.25 (0.71) | 4.32 (0.76) | 6.50 (1.52) |
| Eugonadal-placebo | 6.47 (1.01) | 8.40 (1.05) | 6.30 (0.84) | 6.13 (1.21) | 5.30 (0.87) |
| Hypogonadal | 18.12 (2.91) | 9.56 (2.29) | 11.19 (2.76) | 10.81 (2.92) | 12.06 (2.48) |
| <i>Confusion–bewilderment</i> | | | | | |
| Eugonadal-treated | 6.14 (0.97) | 5.32 (1.27) | 4.04 (0.95) | 3.57 (0.94) | 3.50 (0.87) |
| Eugonadal-placebo | 7.07 (1.11) | 5.73 (1.01) | 5.03 (0.78) | 4.87 (0.98) | 4.60 (0.98) |
| Hypogonadal | 12.25 (1.45) | 8.81 (1.16) | 8.75 (1.11) | 8.56 (1.61) | 8.44 (1.15) |

Standard error of means are given in parentheses.

[$F(4,136)=6.80$, $P<.01$], for depression–dejection [$F(4,136)=7.81$, $P<.01$], for fatigue–inertia [$F(4,136)=4.84$, $P<.01$], for confusion–bewilderment [$F(4,136)=11.96$, $P<.01$] but not for anger–hostility [$F(4,136)=0.79$, ns] or vigor–activity [$F(4,136)=1.54$, ns]. Significant main effects were found for group for tension–anxiety [$F(2,34)=10.83$, $P<.01$], for depression–dejection [$F(2,34)=10.70$, $P<.01$], for anger–hostility [$F(2,34)=27.03$, $P<.01$], for fatigue–inertia [$F(2,34)=8.49$, $P<.01$], for confusion–bewilderment [$F(2,34)=5.80$, $P<.01$] but not for vigor–activity [$F(2,34)=1.28$, ns]. The hypogonadal group reported significantly higher levels of tension–anxiety, depression–dejection, anger–hostility, fatigue–inertia, and confusion–bewilderment than the eugonadal men ($P<.01$). There was a significant Time \times Group interaction for tension–anxiety interaction [$F(8,136)=4.02$, $P<.01$], for anger–hostility [$F(8,136)=2.36$, $P<.05$], for vigor–activity [$F(8,136)=3.84$, $P<.01$], and for fatigue–inertia [$F(8,136)=3.02$, $P<.01$]. The Time \times Group interaction for depression–dejection [$F(8,136)=1.97$, $P=.08$] and for confusion–bewilderment [$F(8,136)=0.61$, ns] was not significant. Planned comparisons showed that significant reduction in tension–anxiety, anger–hostility, and fatigue–inertia by Weeks 1–2 and an increase in vigor–activity by Weeks 3–4 in the hypogonadal group explained the interaction effects, indicating a T treatment effect.

4. Discussion

We found that (trait) cognitive impulsivity (i.e. the tendency to make up one's mind quickly) and (trait) motor impulsivity (i.e. the tendency to act on impulse) accounted for significant amounts of the variance in total aggression levels, over and above age and level of T. This highlights the importance of including individual difference explanatory variables in experimental studies in this area. This finding adds support to an earlier study of violent and nonviolent male parolees by Cherek et al. [31]. They found that violent parolees scored significantly higher on a 'delayed gratification' impulsivity laboratory measure and on the BIS-11 compared to the nonviolent parolees. Furthermore, in another report using the same sample, Cherek et al. [32] found the number of impulsive choices chosen in all parolees to be significantly correlated with the number of aggressive responses reported previously.

Similarly, Galligani et al. [3] found in a study comparing AAS users with a drug-free group that the former reported levels of impulsiveness at least one standard deviation above the mean. The AAS users also reported significantly greater levels of indirect and verbal aggression. Another very recent study found impulsivity to predict AAS using group. That is, young men who report AAS use score significantly higher than non-AAS users [33] on impulsivity and self-reported aggression. Such data, taken as a whole suggest that an underlying mechanism, which inhibits

aggressive behavior, may be less effective, thus contributing to the aetiology of aggressive behavior in normal men. In other words, the link between steroid use and aggression may be that impulsive men are more likely to take steroids and also be aggressive.

However, given the methodological limitations of previous studies (i.e. overreliance of cross-sectional designs), it is difficult to decipher the exact nature of the relationship between impulsivity and aggressive behavior. Deficits in serotonin levels have been implicated in the development of both impulsive and aggressive behavior [34,35]. One recent study [35] found a significant reduction in impulsive responses in a sample of adult males with a history of conduct disorder after administration of D,L-fenfluramine—a drug which releases serotonin (and dopamine). Another found that laboratory aggressive responding increased following the ingestion of a tryptophan (a serotonin precursor)-depleting beverage supporting the notion that serotonin has a role in regulating aggression [36].

We also found that exogenous T administration did not lead to significant increases in a range of aggression measures in eugonadal men. This is not congruent with other experimental studies [15,16]. Most recently, Pope et al. [15] reported a significant increase in both aggressive responding as measured by the PSAP and ratings of manic symptoms. They suggested that the AQ may not be as sensitive as other aggression measures (cf. Ref. [37]) and that the Young Mania Rating Scale [38] and the PSAP were better adapted to detect T effects. This is a moot point given that an earlier paper by the authors [20] reported detecting T treatment effects using the AQ. Moreover, one would have predicted in the present study that our measure of irritability might have at the very least detected any real manic-like related changes. However, it is worth noting that when effect sizes were calculated for Weeks 0–4 comparisons, there were small effects in the direction of an increase in physical aggression. Although, nonsignificant these would need to be borne in mind for future meta-analyses since a series of nonsignificant findings that are all in the same direction may add up to a weak but significant effect when such studies are combined.

It is also reasonable to speculate that the subtle but significant adverse psychological effects reported elsewhere in the literature [16] are only associated with doses of T substantially higher than that employed in this study (>200 mg/week) or utilised in male contraception clinical trials. If this is the case, further high-dose studies are required using detailed self- and partner-reported behavioral and mood assessments in a double-blind, placebo-controlled, cross-over fashion.

We also failed to find any T-related mood effects in the eugonadal-treated group. However, the hypogonadals were identified as the group who benefited most from the T treatment. Significant reductions in tension, anger, and fatigue were reported by Weeks 1–2, followed by a significant increase in vigor by Weeks 3–4. There was also a trend towards a significant Time \times Group interaction for

state self-esteem levels, indicating a marked increase in levels of self-esteem in the hypogonadal group. These findings support earlier research that has found T administration to have positive mood effects when T levels are restored into the normal range [22,39] and suggest that prolonged treatment is likely to maintain these mood benefits, but not further improve them [40]. It is likely the marked elevations in self-esteem scores and positive mood states are concomitant with the restoration of normal sexual function as reported elsewhere [40]. Therefore, it is possible the changes in the psychological functioning are related to changes in sexual function.

The hypogonadal group generally reported significantly elevated levels of verbal aggression, hostility, anger, and irritability compared to both the eugonadal groups. We recognize that a hypogonadal–placebo group has not been included in the design of our study. Ethically and clinically, it would be problematic to treat hypogonadal men with a placebo in order to investigate behavioral changes. However, despite this, it is important to note that the ‘nonblinded’ hypogonadal group did not consistently report lower levels of aggression, hostility, anger, or irritability (comparable to both the eugonadal groups) after treatment commenced. Instead, their self-reported levels remained significantly higher, possibly indicating real concerns as opposed to a placebo effect. To the best of our knowledge, this is the first study to demonstrate the extent of the negative affect experienced by this patient population. These data have implications clinically, for both treatment and therapy.

In conclusion, we have found that supraphysiological levels of T do not lead to significantly increased aggression or mood disturbances. Instead, the inability to control one's behavior when such control is required by a particular situation was found to significantly predict levels of aggression over and above age and T level. Finally, our data for the first time have identified an unacceptably high level of negative affect experienced by hypogonadal patients before and during treatment that requires further investigation.

Acknowledgments

We are grateful to the World Health Organisation (Program No. 96374 to Wu and Archer) for funding this study, to Dr. Helen Buckler and Sister Marie Vickers (Hope Hospital, Manchester, UK) for assisting in the recruitment of hypogonadal patients, and to the Radioimmunoassay Laboratory personnel (Central Manchester Healthcare NHS Trust, UK) for T measurement.

References

- [1] Tricker R, Casaburi R, Storer TW, Clevenger B, Berman N, Shirazi A, Bhasin S. The effect of supraphysiological doses of testosterone on angry behavior in healthy eugonadal men: a clinical research center study. *J Clin Endocrinol Metab* 1996;81:3754–8.
- [2] Parrott AC, Choi PYL, Davies M. Anabolic-steroid use by amateur athletes — effects upon psychological mood states. *J Sports Med Phys Fitness* 1994;34:292–8.
- [3] Galligani N, Renck A, Hansen S. Personality profile of men using anabolic–androgenic steroids. *Horm Behav* 1996;30:170–5.
- [4] Sharp M, Collins D. Exploring the “inevitability” of the relationship between anabolic–androgenic steroid use and aggression in human males. *J Sport Exercise Psychol* 1998;20:379–94.
- [5] Choi PYL, Pope HG. Violence toward women and illicit androgenic–anabolic steroids. *Ann Clin Psychiatry* 1994;6:21–5.
- [6] World Health Organisation Task Force on Methods for the Regulation of Male Fertility. Contraceptive efficacy of testosterone-induced azoospermia in normal men. *Lancet* 1990;336:955–9.
- [7] Rabkin JG, Rabkin R, Wagner G. Testosterone replacement therapy in HIV illness. *Gen Hosp Psychiatry* 1995;17:37–42.
- [8] Tenover JS. Androgen administration to aging men. *Endocrinol Metab Clin North Am* 1994;23:877–92.
- [9] Archer J. The influence of testosterone on human aggression. *Br J Psychol* 1991;82:1–28.
- [10] Aromaki AS, Lindman RE, Eriksson CJP. Testosterone, aggressiveness and antisocial personality. *Aggressive Behav* 1999;25:113–23.
- [11] Virkkunen M, Kallio E, Rawlings R, Tokola R, Poland RE, Guidotti A, Nemeroff C, Bissette G, Kalogeras K, Karonen S, Linnoila M. Personality profiles and state aggressiveness in Finnish alcoholic, violent offenders, fire setters, and healthy volunteers. *Arch Gen Psychiatry* 1994;51:28–33.
- [12] Archer J, Biring SS, Wu FCW. The association between testosterone and aggression among young men—empirical findings and a meta-analysis. *Aggressive Behav* 1998;24:411–20.
- [13] Dabbs JM, Jurkovic GL, Frady RL. Salivary testosterone and cortisol levels among late adolescent male offenders. *J Abnorm Child Psychol* 1991;19:469–78.
- [14] Anderson RA, Bancroft J, Wu FCW. The effects of exogenous testosterone on sexuality and mood of normal men. *J Clin Endocrinol Metab* 1992;75:1503–7.
- [15] Pope HG, Kouri EM, Hudson JI. Effects of supraphysiologic doses of testosterone on mood and aggression in normal men: a randomised controlled trial. *Arch Gen Psychiatry* 2000;57:133–40.
- [16] Su T-P, Pagliaro M, Schmidt PJ, Pickar D, Wolkowitz O, Rubinow DR. Neuropsychiatric effects of anabolic steroids in male normal volunteers. *JAMA, J Am Med Assoc* 1993;269:2760–4.
- [17] Yates WR, Perry PJ, MacIndoe J, Holman T, Ellingrod V. Psychosexual effects of three doses of testosterone cycling in normal men. *Biol Psychiatry* 1999;45:254–60.
- [18] Johnson BT. Software for the meta-analytic review of research literatures. Hillsdale (NJ): Erlbaum, 1989.
- [19] Cherek DR, Schnapp W, Moeller FG, Dougherty DM. Laboratory measures of aggressive responding in male parolees with violent and nonviolent histories. *Aggressive Behav* 1996;22:27–36.
- [20] Kouri EM, Lukas SE, Pope HG, Oliva PS. Increased aggressive responding in male volunteers following the administration of gradually increasing doses of testosterone cypionate. *Drug Alcohol Depend* 1995;40:73–9.
- [21] Bjorkqvist K, Nygren T, Bjorklund A, Bjorkqvist S. Testosterone intake and aggressiveness: real effect or anticipation? *Aggressive Behav* 1994;20:17–26.
- [22] Alexander GM, Swerdloff RS, Wang C, Davidson T, McDonald V, Steiner B, Hines M. Androgen–behaviour correlations in hypogonadal men and eugonadal men. *Horm Behav* 1998;33:85–94.
- [23] Anderson RA, Wu FCW. Comparison between testosterone enanthate-induced azoospermia and oligozoospermia in a male contraceptive study: II. Pharmacokinetics and pharmacodynamics of once weekly administration of testosterone enanthate. *J Clin Endocrinol Metab* 1996;81:896–901.
- [24] Barratt ES. Impulsiveness defined within a systems model of person-

- ality. In: Spielberger ED, Butcher JN, editors. *Advances in personality assessment*, vol. 5. Hillsdale (NJ): Erlbaum, 1985, pp. 13–45.
- [25] Buss AH, Perry M. The Aggression Questionnaire. *J Pers Soc Psychol* 1992;63:452–9.
- [26] O'Connor DB, Archer J, Wu FCW. Measuring aggression: self-reports, partner reports and responses to provoking scenarios. *Aggressive Behav* 2001;27:79–101.
- [27] Heatherton TF, Polivy J. Development and validation of a scale for measuring state self-esteem. *J Pers Soc Psychol* 1991;60:895–910.
- [28] Rathus SA. A 30-item schedule for assessing assertive behavior. *Behav Ther* 1973;4:398–406.
- [29] Buss AH, Durkee A. An inventory for assessing different kinds of hostility. *J Consult Psychol* 1957;21:343–9.
- [30] McNair DM, Lorr M, Droppleman LF. *EdITS manual for the Profile of Mood States*. San Diego (CA): Educational and Industrial Testing Service, 1992.
- [31] Cherek DR, Moeller F, Dougherty DM, Rhoades H. Studies of violent and nonviolent male parolees: II. Laboratory and psychometric measurements of impulsivity. *Biol Psychiatry* 1997;41:523–9.
- [32] Cherek DR, Moeller F, Schnapp W, Dougherty DM. Studies of violent and nonviolent male parolees: I. Laboratory and psychometric measurements of aggression. *Biol Psychiatry* 1997;41:514–22.
- [33] Field C. Personality, aggressive behaviour and anabolic-androgenic steroid use in a sample of men. Unpublished BSc dissertation, University of Leeds, School of Psychology, Leeds, UK, 2001.
- [34] Coccaro EF, Berman ME, Kavoussi RJ, Hauger RL. Relationship of prolactin response to D-fenfluramine to behavioral and questionnaire assessments of aggression in personality disordered men. *Biol Psychiatry* 1996;40:157–64.
- [35] Cherek DR, Lane SD. Fenfluramine effects on impulsivity in a sample of adults with and without history of conduct disorder. *Psychopharmacology* 2000;152:149–56.
- [36] Bjork JM, Dougherty DM, Moeller FG, Cherek DR, Swann AC. The effects of tryptophan depletion and loading on laboratory aggression in men: time course and a food restricted control. *Psychopharmacology* 1999;142:24–30.
- [37] Allen TJ, Dougherty DM, Rhoades HM, Cherek DR. A study of male and female aggressive responding under conditions providing an escape response. *Psychol Rec* 1996;46:651–64.
- [38] Young RC, Biggs JT, Ziegler VE, Meyer DA. A rating scale for mania: reliability, validity and sensitivity. *Br J Psychiatry* 1978;133:429–35.
- [39] Anderson RA, Martin CW, Kung AWC, Everington D, Pun TC, Tan KCB, Bancroft J, Sundaram K, Moo-Young AJ, Baird DT. 7 α -Methyl-19-Nortestosterone maintains sexual behaviour and mood in hypogonadal men. *J Clin Endocrinol Metab* 1999;84:3556–62.
- [40] Wang C, Alexander G, Berman N, Salehian B, Davidson T, McDonald V, Steiner B, Hull L, Callegari C, Swerdloff RS. Testosterone replacement therapy improves mood in hypogonadal men—a clinical research center study. *J Clin Endocrinol Metab* 1996;81:3578–83.

AA2500 Testosterone Gel Normalizes Androgen Levels in Aging Males with Improvements in Body Composition and Sexual Function

C. STEIDLE, S. SCHWARTZ, K. JACOBY, T. SEBREE, T. SMITH, R. BACHAND, AND THE NORTH AMERICAN AA2500 T GEL STUDY GROUP

Northeast Indiana Research (C.S.), Fort Wayne, Indiana 46825; Diabetes and Glandular Disease Research Associates (S.S.), San Antonio, Texas 78229; Integrity Medical Research (K.J.), Seattle, Washington 98133; and Auxilium Pharmaceuticals, Inc. (T.Se., T.Sm., R.B.), Norristown, Pennsylvania 19041

Testosterone replacement in hypogonadal men improves body composition, mood, and sexual functioning. In this 90-d study, we compared the pharmacokinetics and treatment effectiveness of a topical testosterone gel (AA2500) at two concentrations, 50 mg/d and 100 mg/d, to a testosterone patch and placebo gel in 406 hypogonadal men. Pharmacokinetic profiles were obtained, body composition was measured, and mood and sexual function were monitored. AA2500 treatments resulted in dose-dependent improvements in all pharmacokinetic parameters, compared with testosterone patch and placebo. Mean average concentrations at d 90 T were 13.8, 17.1, 11.9, and 7.3 nmol/liter for 50 mg/d AA2500, 100 mg/d AA2500,

testosterone patch, and placebo, respectively. At d 90, the 100 mg/d AA2500 treatment improved lean body mass by 1.7 kg and percentage of body fat by 1.2% to a significantly greater degree than either control treatment. Significant improvements in spontaneous erections, sexual desire, and sexual motivation were also evidenced with the 100 mg/d AA2500 dose in comparison with placebo. Testosterone gel was well tolerated; however, the testosterone patch resulted in a high rate of application site reactions. Overall, AA2500 is an effective, well tolerated treatment for hypogonadism. (*J Clin Endocrinol Metab* 88: 2673–2681, 2003)

THE USE OF testosterone (T) replacement therapy in hypogonadal men has been well documented. Specifically, restoration of serum T concentrations to within normal limits (*i.e.* similar to that of eugonadal men) can maintain sexual characteristics, sexual behavior, energy, mood, and muscle development and improve bone density (1). Currently there are a number of different T dosage forms available for replacement therapy in hypogonadal men, but many of these formulations have limitations. Orally available T is relatively insoluble and subject to a high first-pass effect in the liver. Intramuscular depot injections are used widely for replacement therapy but are inconvenient and result in wide fluctuations in T levels. Specifically, high initial peak levels, followed by serum T levels below the lower limit of normal toward the end of the cycle, lead to a return of clinical signs and symptoms.

The skin and oral mucosa are considered favorable routes for the delivery of T. Transdermal T patches, including the scrotal patch (Testoderm), the nonscrotal permeation-enhanced patch with an alcohol-based reservoir (Androderm), and the nonscrotal patch without a reservoir (Testoderm TTS) provide a more consistent delivery of T into the systemic circulation, although serum T levels are not always

maintained within normal limits over a 24-h period. Long-term use of these patches (3–10 yr) has been shown to be effective in maintaining sexual function and bone and muscle mass in both young and elderly hypogonadal males (2–5); however, skin tolerability problems or the need for shaving large areas of scrotal skin invariably affect compliance with transdermal patches. Skin reactions commonly occur at the patch application site, particularly with the permeation-enhanced T patches causing erythema or pruritus. Blister reactions also occur leading to scarring and discontinuation of treatment (6, 7).

Previously, it has been reported that a T gel (AndroGel), when applied over a larger area of skin, can achieve serum T levels in the normal range and produce less skin irritation than T patches. A new, unique topical T gel formulation (AA2500) has been designed to provide consistent transdermal absorption of T over 24 h after a single dose and is hereby reported. Before this study, the pharmacokinetic (PK) profile of this new T gel (AA2500) was compared with AndroGel. Data have demonstrated that after topical application of a single dose of AA2500 T gel or AndroGel, the time to maximum concentration (T_{max}) was comparable between the two formulations indicating no appreciable differences in the rate of absorption. However, the 0- to 24-h area under the curve (AUC_{0-24}) and maximum concentration (C_{max}) were consistently higher following application of AA2500 with approximately 30% higher serum T levels being noted. The safety profile of these two topical gel formulations was similar (8).

The study reported here involves comparisons among four parallel treatment groups in 406 patients consisting primarily of aging males with low serum T and associated signs and

Abbreviations: AUC_{0-24} , 0- to 24-h Area under the curve; BPH, benign prostatic hyperplasia; C_{avg} , mean concentration; C_{max} , maximum concentration; C_{min} , minimum concentration; DHT, dihydrotestosterone; DRE, digital rectal examination; %F, percentage fat; FM, fat mass; HDL, high-density lipoprotein; I-PSS, International Prostate Symptom Score; LBM, lean body mass; LDL, low-density lipoprotein; PK, pharmacokinetic; PSA, prostate-specific antigen; T, testosterone; TBM, total body mass; TC, total cholesterol.

symptoms of hypogonadism. Two doses of AA2500 T gel (50 mg/d and 100 mg/d) were compared with a T patch treatment (Androderm, two patches delivering 5 mg T daily), a dose that is known to give rise to clinically meaningful increases in serum T levels with amelioration of signs and symptoms (9). The fourth parallel group was a matching placebo gel to provide a blinded comparator for the two doses of AA2500 T gel, which also provided a valid overall assessment of clinical and subjective symptom improvement. In this 90-d study, periodic 24-h PK profiles of total T and dihydrotestosterone (DHT) were obtained, and the effect of normalizing serum T on body composition, sexual function, mood, and bone mineral density were assessed. Routine safety evaluations were conducted, including skin irritation assessments at the study drug application site. As such, this study design was robust and unique in the assessment of the efficacy of topical transdermal T in normalizing serum T levels, ameliorating signs and symptoms of hypogonadism, and assessing its safety.

Subjects and Methods

Subjects

Four hundred six male patients were randomized and treated at 43 clinics in the United States. Approximately 100 patients were randomized to each treatment group (Table 1). Patients were between 20 and 80 yr of age and had a morning T level of 10.4 nmol/liter or less at screening (measured at a central laboratory) and one or more symptoms of low T (*i.e.* fatigue, decreased muscle mass, reduced libido, reduced sexual functioning of a nonmechanical nature). Except for hypogonadism, the patients were in generally good health as evidenced by medical history; complete physical examination including a digital rectal examination (DRE), 12-lead electrocardiogram, vital sign assessments, clinical laboratory and urinalysis assessments, prostate assessment [International Prostate Symptom Score (I-PSS)]; and normal tests for prostate-specific antigen (PSA), hepatitis, and drugs of abuse. If the patient was receiving lipid-lowering agents, anxiolytics, lithium, antidepressants, hypnotics, antipsychotics, α_1 blockers, or herbal treatments for benign prostatic hyperplasia (BPH), the dose had to have been stable for at least 3 months before entering the study. Patients were excluded from the study if they had any generalized skin irritation or disease that might have interfered with androgen absorption; had received any estrogen therapy, an LHRH antagonist, human GH therapy; or had a history of drug abuse within

12 months. Also excluded were patients who had used either Viagra or apomorphine within 30 d or were treated with T or anabolic supplements within 6 wk before the study. The study was conducted in accordance with the Declaration of Helsinki and complied with Good Clinical Practice, and all patients signed an informed consent agreement previously approved by one of the participating institutional review boards.

Study drugs

AA2500 T gel (Testim) was supplied by Auxilium Pharmaceuticals, Inc. (Norristown, PA). The four daily treatments under study were 50 mg/d AA2500 or 100 mg/d AA2500, matching placebo gel, and a transdermal T patch (Androderm, two patches \times 2.5 mg T), each containing 12.2 mg T. The AA2500 and placebo gel were identical and applied as two tubes of 50 mg T (100 mg/d), one tube of 50 mg T and one tube of placebo (50 mg/d), or two tubes of placebo. Neither the patients nor the investigators were aware of the contents of the tubes.

All study drug treatments were applied in the morning; repeat applications occurred at the same time of day for the duration of the study. Each day in the gel-treated group, patients applied the contents of two tubes. The content of one tube was applied to one shoulder and the content of the remaining tube was applied to the other shoulder. Patients allocated to receive the T patch applied two adhesive patches daily. Application sites included the back, abdomen, upper arms, and thighs. Patches were to be worn for 24 h and then replaced each morning at approximately the same time.

Study design

The study was designed as a randomized, multidose, multicenter, active, and placebo-controlled study. Patients were randomized to 50 mg AA2500 T gel (99 patients) or 100 mg AA2500 T gel (106 patients), matching placebo gel (99 patients), or T patch (102 patients). Randomization was performed to ensure an equal distribution of treatments across study centers. The study was double blinded for the AA2500 and placebo groups and open label for the T patch group. Patients randomized to one of the two AA2500 arms could be titrated at d 60 based on their d 30 T PK profile. Patients were titrated from 50 mg/d to 100 mg/d at d 60 if their d 30 mean serum T concentration (C_{avg}) was less than 10.4 nmol/liter (300 ng/dl). Patients were titrated from 100 mg/d to 50 mg/d at d 60 if their d 30 C_{avg} was more than 34.7 nmol/liter (1000 ng/dl). These titration decisions were undertaken by a third-party physician who was unaware of any clinical aspects of the individual patients and not otherwise involved in the study.

On d -1, patients had a baseline 24-h profile for serum T and DHT consisting of serum samples taken at 0800, 1000, and 1200 h, and 1600

TABLE 1. Subject characteristics

| | AA2500 | | T patch | Placebo | Total |
|-------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | 50 mg/d | 100 mg/d | | | |
| Demographics | | | | | |
| n | 99 | 106 | 102 | 99 | 406 |
| Age (yr) | 58.1 \pm 9.7 | 56.8 \pm 10.6 | 60.5 \pm 9.7 | 56.8 \pm 10.8 | 58.0 \pm 10.3 |
| Height (cm) | 178 \pm 6 | 178 \pm 8 | 178 \pm 6 | 180 \pm 7 | 179 \pm 7 |
| Weight (kg) | 95.7 \pm 13.4 | 95.7 \pm 14.4 | 95.1 \pm 13.5 | 98.5 \pm 15.6 | 96.2 \pm 14.2 |
| BMI | 30.0 \pm 3.7 | 29.9 \pm 3.3 | 29.9 \pm 3.8 | 30.3 \pm 3.8 | 30.0 \pm 3.6 |
| T (nmol/liter) ^a | 8.1 \pm 2.0 | 8.1 \pm 2.2 | 8.3 \pm 2.4 | 7.9 \pm 2.8 | 8.1 \pm 2.3 |
| I-PSS score | 6.5 \pm 6.0 | 4.8 \pm 5.0 | 6.2 \pm 5.5 | 5.0 \pm 5.3 | 5.6 \pm 5.5 |
| PSA (ng/ml) | 1.17 \pm 0.89 | 1.29 \pm 0.96 | 1.45 \pm 1.18 | 1.13 \pm 1.00 | 1.26 \pm 1.02 |
| Cause of hypogonadism ^b | | | | | |
| Primary (n) | 8 | 7 | 4 | 3 | 22 |
| Secondary (n) | 91 | 98 | 98 | 95 | 382 |
| Aging (%) ^c | 70.7 | 58.1 | 66.7 | 61.2 | 64.1 |
| Normogonadotrophic (%) ^c | 19.2 | 30.5 | 26.5 | 31.6 | 27.0 |

Demographic values are expressed as means \pm 1 SD. BMI, Body mass index.

^a 0800 h serum concentration at screening examination.

^b Two subjects had a missing cause of hypogonadism.

^c Percentage of total by treatment group. Some subjects had more than one symptom, but all were required to have at least one. Distribution by cause is shown only if it occurred in $\geq 4\%$ of subjects.

and 0800 h on d 1, immediately before the first dose of study drug. On d 30 and 90, patients had a 24-h profile for T and DHT consisting of serum samples at predose and 2, 4, 8, 12, and 24 h after study drug administration. On d 60, a single 0800-h serum sample was taken for T and DHT. Blood samples for clinical laboratory assessment were collected at screening and on d -1, 30, 60, and 90. The prostate was evaluated at screening (PSA levels only) and d -1 and 90 with PSA levels, I-PSS, and DRE. Body composition [total body mass (TBM), lean body mass (LBM), and fat mass (FM)] and bone mineral density of the L1-L4 section of the lumbar spine were measured by dual energy x-ray absorptiometry on d -1 and d 90. Percentage fat (%F) was derived from FM and TBM. All body composition and bone mineral density measurements were centrally monitored and analyzed by Synarc, Inc. (Maynard, MA). Sexual function and mood questionnaires were recorded daily for 14 d before d 1 and daily for 7 d before d 30, 60, and 90. Data were collected centrally in real time via an interactive voice response system using the telephone. Skin irritation examination using a standardized, discrete scoring system was performed at d 1 (before dosing), 30, 60, and 90. Medical history and physical exams were completed, and all adverse events were recorded.

Methods

The skin irritation scoring was based on the following schema: 0, no erythema; 1, minimal erythema; 2, moderate erythema with sharply defined borders; 3, intense erythema with or without edema; 4, intense erythema with edema and blistering.

Sexual functioning and mood assessments were based on a questionnaire, one that had been validated for assessment of sexual function and mood and used previously in the evaluation of the effects of T gel on sexual function and mood (2). The questionnaire elicited information on sexual functions: performance, motivation, spontaneous erections, desire, enjoyment (with and without a partner), and satisfaction with erection duration and size. The sexual performance assessment was based on the following activities: orgasm, ejaculation, intercourse, masturbation, and erection in response to a sexual activity. The sexual performance score was the average number of days per 7-d week of these five activities. The sexual motivation assessment was based on the following activities: sexual daydreams, anticipation of sex, sexual interaction with partner, flirting by subject, and flirting by others toward subject. The sexual motivation score was the average number of days per 7-d week that these five activities occurred. The evaluation of spontaneous erections was the average number of days in a 7-d week that either spontaneous nighttime or daytime erections occurred. Sexual desire, sexual enjoyment, and satisfaction with erection were assessed on a Likert-type scale (score 0 to 7) and were calculated as average scores. Percentage of full erection was scored from 0% to 100%. Patients also rated positive mood (alert, full of energy, friendly, well or good) and negative mood (angry, irritable, sad or blue, tired, nervous) on a 0 to 7 categorical scale (0 = not at all true to 7 = very true). Average daily scores were computed.

Serum T and DHT levels were all measured at ICON Laboratories (Farmingdale, NY), using validated RIA kits. Kits (Diagnostic Products, Los Angeles, CA) were used for the T assays and kits obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX) were used for DHT assays. The lower limits of detection for the T and DHT pharmacokinetic assays were 0.1 nmol/liter (4 ng/ml) and 0.01 nmol/liter (4 pg/ml), respectively. The DHT assay had a 0.02% or less cross-reactivity (after solvent extraction) with T and T had a 3.3% or less cross-reactivity with DHT up to 173.6 nmol/liter (5000 ng/dl). The mean accuracy (recovery) of T determined by spiking steroid-free serum with varying amounts of T [1.5–1.3 nmol/liter (44–36 ng/dl)] was 98% (range, 93–103). The intra- and interassay coefficients of the T assay were 6.7% and 7.9% for a control group adult male range of 8.5–63.8 nmol/liter (245–1836 ng/dl). The mean accuracy (recovery) of DHT determined by spiking steroid-free serum with various amounts of DHT [0.2–1.4 nmol/liter (59–418 pg/ml)] was 94% (range, 85–130). The intra- and interassay coefficients of the DHT assay were 4.6% and 6.4%, respectively, for a control group adult male range of 0.3–2.4 nmol/liter (97–711 pg/ml).

Statistical analyses

The 24-h PK profiles for T and DHT were summarized by C_{avg} (AUC_{0-24} divided by the 24-h sampling period, where AUC is cal-

culated using the trapezoidal rule), the minimum postdose concentration (C_{min}), and the postdose C_{max} . The changes from baseline to d 30 and d 90 in C_{min} , C_{avg} , and C_{max} were analyzed using an analysis of covariance with baseline value as the covariate and treatment group as the factor. Patients randomized to AA2500 (50 mg/d or 100 mg/d) may have had their dose changed at the d 60 visit. Those patients with a dose change will be analyzed at d 90 using the dose they received at the d 60 visit. Similar analyses were used for the change from baseline in sexual function, mood, and body composition as well as for the clinical laboratory parameters at d 30, 60, and 90. Treatment-emergent adverse events were compared using a Fisher's exact test. Skin irritation at d 30, 60, and 90 was analyzed using a Wilcoxon rank sum test. At d 30 and 90, the 50 mg/d and 100 mg/d AA2500 treatment groups were compared with T patch (PK parameters, sexual function, body composition, and mood) and placebo (sexual function, body composition, and mood). For each comparison, including safety parameters, an α -value of 0.05 was considered significant. The changes from baseline in sexual function, body composition, and mood were also analyzed for nonzero differences within each treatment group based on the adjusted least squares means from the analysis of covariance model. SAS version 6.12 (SAS Institute, Inc., Cary, NC) was used for all analyses. All data in tables are presented as means (\pm sd).

Results

Subjects

A total of 406 patients were randomized with 99, 106, 102, and 99 being randomized to the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively (Table 1). Baseline patient characteristics (age, height, weight, body mass index, serum T at screening, I-PSS scores, and PSA levels) were comparable. Of the patients who had a valid PK profile, 70 of 399 (17.5%, 21 patients in 50 mg/d AA2500, 20 patients in 100 mg/d AA2500, 16 patients in T patch, and 13 patients in placebo groups) had a C_{avg} above 10.4 nmol/liter at baseline. Of these patients, 71% had at least one or more serum T measurement less than 10.4 nmol/liter during the course of d -1. Baseline mean C_{avg} serum T concentrations were 12.6, 12.1, 12.8, and 12.4 nmol/liter in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively. Fifty percent of the patients were aged 58 yr or older and approximately 26% were aged 65 yr and older with a mean age of 58 yr. Patient hypogonadism was primarily attributed to the secondary cause of aging and normogonadotrophic hypogonadism; these cases accounted for 91% of all causes in the overall population (Table 1). A significant proportion of enrolled patients completed the 90-d study (90% and 92% in the AA2500 and placebo groups, respectively, and 75% in the T patch group). The primary reason for the higher rate of discontinuations in the T patch group was adverse events (17%) with the majority of events being related to skin irritations at the patch site. Titrations made at d 60 in the AA2500 T gel groups were: 52 patients started on 50 mg/d dose and remained on 50 mg/d dose for the entire study, 43 patients who started on 50 mg/d dose were titrated up to the 100 mg/d dose, 93 patients who started on 100 mg/d dose remained at the 100 mg/d dose for the entire study, and 4 patients who started on 100 mg/d dose were titrated down to 50 mg/d dose. Patients who remained at 50 mg were more likely to have secondary hypogonadism, excluding aging, than patients who titrated to 100 mg. Dosing compliance ranged from 94.9% (placebo) to

97.1% (50 mg/d AA2500 and 100 mg/d AA2500 combined analysis).

T pharmacokinetics (Fig. 1 and Table 2)

At baseline, mean C_{avg} serum T concentrations were below the normal adult range (10.4–34.7 nmol/liter) and similar across treatment groups. By d 30, the mean C_{avg} for the 50 mg/d AA2500 treatment had increased 50% over baseline with a similar increase being evidenced in the T patch treatment group. The 100 mg/d AA2500 dose resulted in a 173% increase with a significant difference ($P < 0.001$) in comparison with the T patch treatment group. The C_{avg} was increased above 10.4 nmol/liter in 55% of 50 mg/d AA2500 patients, 95% of the 100 mg/d AA2500 patients, 68% of the T patch patients, and 8% of the placebo patients. In the 100

mg/d AA2500 group, 30 patients had a C_{max} higher than the upper limit of normal (34.7 nmol/liter), but 26 of these patients had a C_{avg} still between 10.4–34.7 nmol/liter. The degree of fluctuation during a day in serum values [$(C_{max} - C_{min})/C_{avg}$] was significantly smaller in the two AA2500 dose groups in comparison with the T patch group. By d 90, similar results were seen across the treatment groups. Approximately 75% of 50 mg/d AA2500 and 80% of 100 mg/d AA2500 treated patients had C_{avg} values above 10.4 nmol/liter, in comparison with 57% of the T patch-treated patients and 10% of placebo-treated patients.

DHT pharmacokinetics (Fig. 2 and Table 3)

At baseline, mean C_{avg} serum DHT concentrations were below the normal adult male range (0.9–2.6 nmol/liter) and similar across treatment groups. Mean changes in DHT C_{avg} from baseline to d 30 for the 50 mg/d AA2500 and 100 mg/d AA2500 dose groups were more than 4- and 7-fold greater, respectively, than changes observed in the T patch treatment group ($P < 0.001$ for each comparison). Similar to C_{avg} , C_{min} results further demonstrated the effectiveness of both AA2500 doses in increasing the d 30 C_{min} to a significantly greater degree than the T patch treatment ($P < 0.001$ for each comparison). The 100 mg/d AA2500 dose achieved a mean d 30 C_{min} that was within the normal range. For C_{max} , the d 30 effects reported were similar to that observed with C_{avg} . The C_{max} mean changes in serum DHT from baseline to d 30 for both AA2500 dose groups were approximately 4- and 7-fold greater than that evidenced in the T patch group ($P < 0.001$ for each comparison) with 15 (16%), 39 (42%), 1 (1%), and no patients in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively, exceeding the normal range. At d 30, examination of the DHT to T C_{avg} ratio demonstrated that this ratio was not altered by either the T patch or placebo treatment, whereas a near doubling of the ratio was, respectively, evidenced with both AA2500 doses. By d 90, similar results were seen across the treatment groups. Additionally, the higher serum levels of DHT obtained with the AA2500 treatments correlated with the serum T levels obtained.

Body composition (Fig. 3)

At baseline, there were no significant differences in LBM, FM, %F, and TBM among the four treatment groups. At d 90, the 100 mg/d AA2500 treatment increased LBM to a significantly greater degree than the T patch or placebo treatment ($P < 0.05$ for each comparison) with mean changes from baseline of 1.5 ± 4.5 , 1.7 ± 2.6 , 0.9 ± 1.8 , and 0.6 ± 1.8 kg for the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively. With the exception of placebo treatment, all treatments resulted in a decrease in FM, which were significant, compared with placebo ($P < 0.01$). Reductions of 0.8 ± 2.4 , 0.8 ± 2.0 , 0.4 ± 1.8 , and 0.1 ± 1.5 kg were noted in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively. Reductions in %F were evidenced in all treatment groups, with the AA2500 treatments yielding the most notable decreases. Specifically, at d 90, the 50 mg/d AA2500 dose resulted in a reduction of $1.1 \pm 3.2\%$ which was sig-

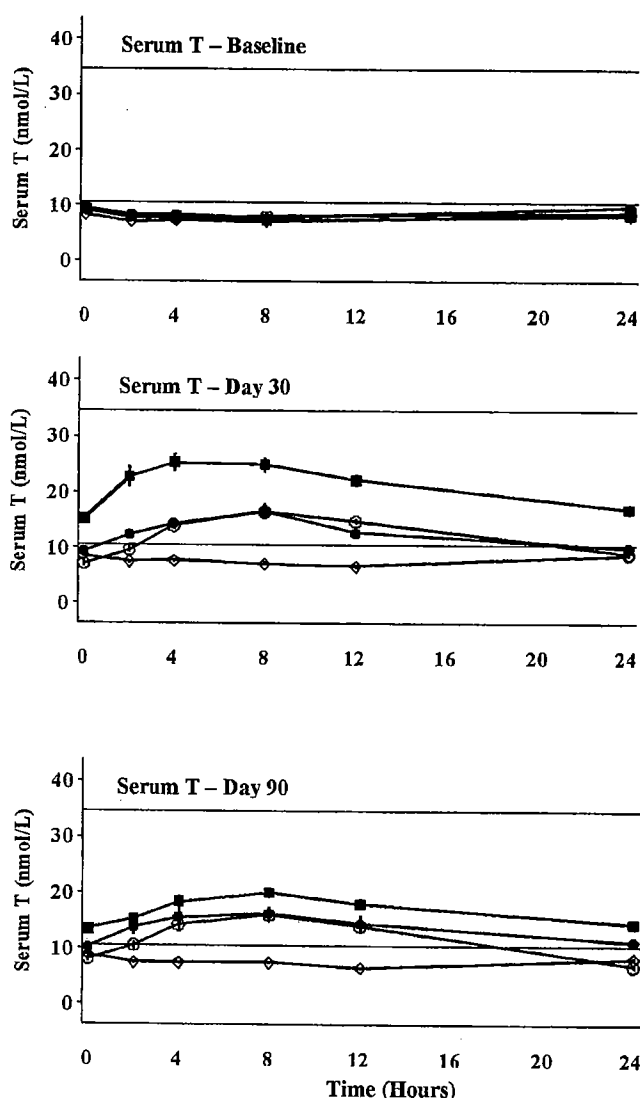


FIG. 1. Serum T concentrations before (baseline) and after study drug treatment on d 30 and 90. Time 0 was approximately 0800 h. Solid horizontal lines denote the adult male range (10.4–34.7 nmol/liter). ●, 50 mg/d AA2500; ■, 100 mg/d AA2500; ○, T patch; ◇, placebo.

TABLE 2. T (nmol/liter): mean d 30 and 90

| | | AA2500 | | T patch | Placebo |
|--------|------------------|----------|--------------------------|--------------------------|------------|
| | | 50 mg/d | 100 mg/d | | |
| Day 30 | | | | | |
| | C _{avg} | Baseline | 8.6 ± 2.8 | 7.8 ± 2.8 | 8.2 ± 2.8 |
| | | Actual | 12.7 ± 6.5 | 21.3 ± 9.9 ^b | 12.7 ± 4.2 |
| | C _{min} | Baseline | 6.8 ± 2.4 | 6.2 ± 2.6 | 6.7 ± 2.3 |
| | | Actual | 7.7 ± 4.4 ^a | 13.6 ± 6.5 ^b | 6.2 ± 2.9 |
| | C _{max} | Baseline | 10.7 ± 3.6 | 9.9 ± 3.2 | 10.2 ± 3.7 |
| Day 90 | | Actual | 18.8 ± 12.9 ^a | 31.2 ± 19.8 ^b | 18.8 ± 6.9 |
| | C _{avg} | Baseline | 9.2 ± 3.4 | 7.7 ± 2.4 | 8.3 ± 2.8 |
| | | Actual | 13.8 ± 8.1 | 17.1 ± 8.2 ^b | 11.9 ± 4.6 |
| | C _{min} | Baseline | 7.4 ± 2.8 | 6.1 ± 2.3 | 6.7 ± 2.1 |
| | | Actual | 8.7 ± 3.9 ^b | 10.9 ± 6.0 ^b | 5.7 ± 2.8 |
| | C _{max} | Baseline | 11.3 ± 4.1 | 9.8 ± 2.9 | 10.3 ± 3.7 |
| | | Actual | 19.5 ± 12.2 | 24.4 ± 13.8 ^b | 18.5 ± 8.2 |

Values are expressed as means ± 1 SD. Change from baseline significant vs. T patch: ^a $P < 0.05$, ^b $P < 0.001$.

nificant in comparison to the reduction evidenced with placebo treatment ($0.2 \pm 1.4\%$, $P < 0.05$). Furthermore, the 100 mg/d AA2500 treatment resulted in a $1.2 \pm 1.9\%$ reduction at d 90, which was not only significant in comparison with placebo ($P < 0.01$) but also significant in comparison with the T patch treatment ($0.5 \pm 1.6\%$, $P < 0.05$). Although all treatments resulted in minimal increases in TBM, no significant differences were noted among the treatment groups.

Mood and sexual function (Table 4)

Although all treatments resulted in mean improvements from baseline in both positive and negative mood scores, no significant differences among the treatment groups were observed.

At baseline, sexual function scores were similar across the four treatment groups. Evaluation of the mean data demonstrated that the 100 mg/d AA2500 dose showed a significant improvement at d 90 over placebo treatment for spontaneous erections ($P < 0.001$), sexual motivation ($P < 0.05$), sexual desire ($P < 0.01$), and sexual performance ($P < 0.05$). Furthermore, the improvement from baseline was also significant for these parameters.

All other measures of sexual function (e.g. sexual enjoyment with a partner, sexual enjoyment without a partner, satisfaction with erection duration, and percentage of full erection) showed no significant difference in improvement between treatment groups.

Safety

Adverse events. The incidence of treatment-related adverse events was 29.1%, 36.9%, 62.7%, and 40.4% in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively. Although treatment in the AA2500 and placebo groups was relatively well tolerated over the 90-d study period, the T patch-treated patients experienced a substantially higher rate of adverse events. Those most commonly seen were application site erythema, application site rash, application site pruritus, application site reactions, and application site irritation.

Specific events that were possibly or probably related to study drug and reported by 1% or more of the AA2500

patients and greater than placebo included application site reactions, BPH, increases in blood pressure and hematocrit/hemoglobin, gynecomastia, headache, hot flushes, insomnia, increased lacrimation, mood swings, smell and taste disorders, and spontaneous penile erections.

Only six patients in AA2500 treatment groups experienced adverse events that led to discontinuation. Specific events in the 100 mg/d AA2500 treatment group included vertigo, coronary artery disease, depression with suicidal ideation, urinary tract infection/pneumonia, and hypertension. All events with the exception of hypertension were considered unrelated to treatment. Mood swings, considered related to treatment, was the only event in the 50 mg/d AA2500 treatment group that led to discontinuation. Lastly, no patients in the 50 mg/d or 100 mg/d AA2500 treatment groups discontinued because of skin reaction, whereas the majority of patients that discontinued in the T patch group did so as a result of local dermal site reactions ($n = 15$).

With regard to prostate-related events, mild BPH was reported in two patients in the 100 mg/d AA2500 treatment group and one patient in the placebo treatment group. Additionally, two T patch-treated patients were diagnosed with prostatic cancer.

Laboratory analyses

Statistically significant differences between the 50 mg/d and/or 100 mg/d AA2500 groups and placebo groups in serum blood urea nitrogen (-2.0 ± 4.0 , -1.7 ± 4.3 , and -0.6 ± 3.9 mg/dl, respectively), creatinine (0.04 ± 0.12 , 0.07 ± 0.14 , and -0.02 ± 0.12 mg/dl, respectively), and fasting glucose levels (-2.2 ± 18.1 , -5.6 ± 25.1 , and 4.0 ± 25.3 mg/dl, respectively) were observed; however, these differences were minor and not clinically meaningful.

At d 90, clinically notable decreases from baseline in average total cholesterol (TC), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were evidenced with the 100 mg/d AA2500 group (-7% , -7% , and -8% , respectively). Mean d 90 LDL/HDL ratios (2.73, 2.56, 2.52, and 2.41 for the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively) remained essentially unchanged from baseline. Additionally, mean d 90 HDL/TC

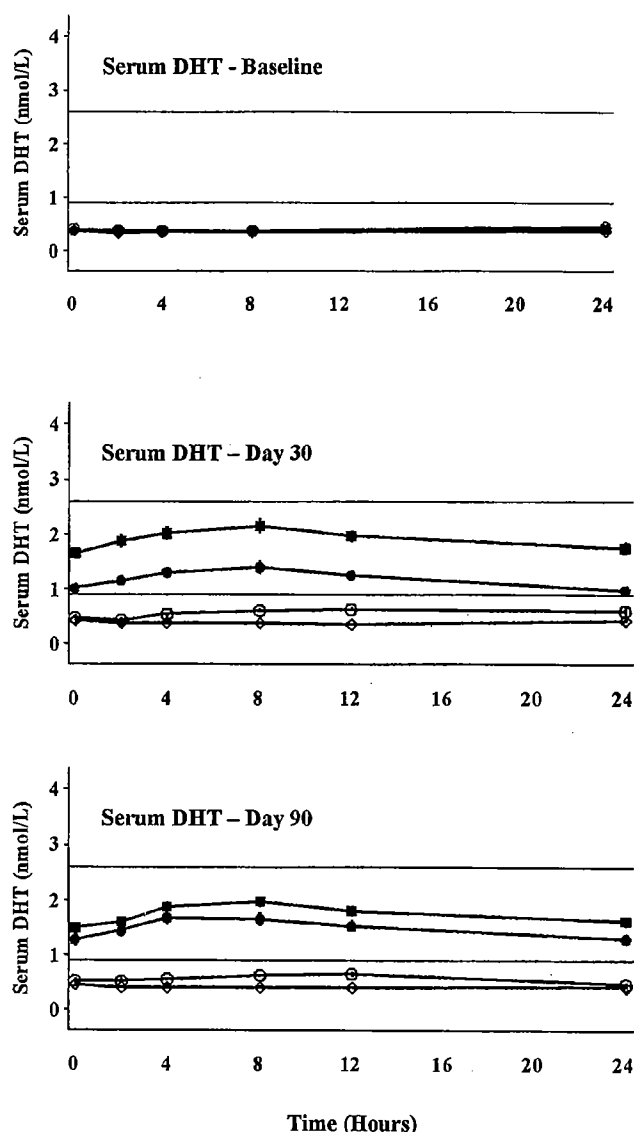


FIG. 2. Serum DHT concentrations before (baseline) and after study drug treatment on d 30 and 90. Time 0 was approximately 0800 h. Solid horizontal lines denote the adult male range (0.8–2.6 nmol/liter). ●, 50 mg/d AA2500; ■, 100 mg/d AA2500; ○, T patch; ◇, placebo.

ratios (0.23, 0.24, 0.24, and 0.24 for the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively) also remained unchanged.

Increases in hemoglobin and hematocrit are known pharmacological class effects of T. Consistent with this, patients in the 50 mg/d and 100 mg/d AA2500 group experienced statistically significant mean d 90 increases in hematocrit and hemoglobin of $2.3 \pm 3.4\%$ and 0.96 ± 0.96 g/dl, respectively, in the 50 mg/d AA2500 group and $2.8 \pm 3.5\%$ and 0.94 ± 1.06 g/dl in the 100 mg/d AA2500 group, compared with the placebo treatment group ($-0.1 \pm 2.8\%$ and 0.12 ± 0.71 g/dl) and T patch treatment group ($1.1 \pm 2.6\%$ and 0.48 ± 0.74 g/dl). The effects observed with T patch treatment were consistently greater than observed with placebo treatment

but less than those observed with the AA2500 treatments, reflecting the lower average serum T levels associated with the T patch treatment. At d 30 and 60, similar effects were reported for the AA2500 treatment group. Overall, approximately 3%, 6%, 1%, and 1% of patients in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively, experienced a hematocrit value more than 55% at least once during the study. Statistically significant mean changes were also reported for lymphocytes and monocytes, but the changes were small and not of apparent clinical significance.

PSA values (Table 5) more than 4.0 ng/ml were noted at least once during treatment in 1.8%, 2.9%, 6.6%, and 3.2% of patients in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively. PSA elevations were noted in all groups with the T patch group evidencing the greatest number of transient and persistent elevations. Mean changes from baseline to d 90 of 0.3 ± 1.8 , 0.1 ± 0.4 , 0.2 ± 0.6 , and -0.1 ± 0.4 ng/ml for the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo treatment groups, respectively. Of note, one patient in the 50 mg/d AA2500 treatment group experienced a transient elevation of 17.6 ng/ml (without clinically valid explanation) that upon repeat evaluation returned to normal (3.6 ng/ml).

The changes from baseline for I-PSS were small, and the incidence of patients experiencing a general worsening of their DRE was low (3.4%, 1.4%, 0%, and 4.2% in the 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo groups, respectively). Both parameters provided no evidence of clinically relevant treatment-related effects or differences.

Skin irritation (Fig. 4)

Figure 4 provides a graphic illustration of the frequency distribution of patients having positive skin irritation scores. It can be seen that the events occurred predominantly in the T patch treatment group and only a few mild reactions occurred in the combined AA2500 and placebo treatment groups. Additionally, the figure illustrates that the T patch acted as an irritant in some patients who experienced classic signs of contact dermatitis and that the AA2500 treatments were no more irritating than the placebo gel vehicle from d 60 through the completion of the study.

Discussion

This study demonstrated that this new, unique T gel (AA2500), when titrated to clinical effectiveness, was superior to the T patch in normalizing serum T in patients with hypogonadism. Specifically, the 100 mg/d dose was not only effective in significantly improving sexual performance, sexual motivation, and sexual desire and increasing spontaneous erections but also increasing LBM and decreasing FM and %F. There were associated increases in hemoglobin and hematocrit, which are known pharmacological class effects of T administration. The small mean increases in PSA observed with AA2500 doses and the T patch treatment groups, but not the placebo group, were not associated with an increase in I-PSS. Small decreases in TC, LDL, and HDL were observed in the AA2500 treatment groups with no changes in HDL/TC or LDL/HDL ratios. Erythema, rash, pruritus,

TABLE 3. DHT (nmol/liter): mean d 30 and 90

| | | AA2500 | | T patch | Placebo |
|-----------|-----------|-------------------|-------------------|-----------------|-----------------|
| | | 50 mg/d | 100 mg/d | | |
| Day 30 | C_{avg} | Baseline | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.2 |
| | Actual | 1.2 ± 0.7^a | 1.9 ± 1.0^a | 0.6 ± 0.3 | 0.4 ± 0.2 |
| C_{min} | Baseline | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 |
| | Actual | 0.8 ± 0.6^a | 1.4 ± 0.9^a | 0.4 ± 0.2 | 0.3 ± 0.2 |
| C_{max} | Baseline | 0.5 ± 0.2 | 0.5 ± 0.3 | 0.5 ± 0.3 | 0.5 ± 0.2 |
| | Actual | 1.7 ± 1.0^a | 2.6 ± 1.4^a | 0.8 ± 0.7 | 0.5 ± 0.3 |
| DHT/T† | Baseline | 0.05 ± 0.02 | 0.05 ± 0.03 | 0.05 ± 0.02 | 0.06 ± 0.04 |
| | Actual | 0.09 ± 0.04^a | 0.09 ± 0.04^a | 0.05 ± 0.02 | 0.06 ± 0.04 |
| Day 90 | C_{avg} | Baseline | 0.5 ± 0.2 | 0.4 ± 0.2 | 0.4 ± 0.2 |
| | Actual | 1.5 ± 0.7^a | 1.8 ± 0.9^a | 0.6 ± 0.3 | 0.4 ± 0.2 |
| C_{min} | Baseline | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| | Actual | 1.0 ± 0.6^a | 1.2 ± 0.7^a | 0.3 ± 0.2 | 0.3 ± 0.2 |
| C_{max} | Baseline | 0.6 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.2 | 0.5 ± 0.2 |
| | Actual | 2.0 ± 0.9^a | 2.3 ± 1.2^a | 0.8 ± 0.4 | 0.5 ± 0.3 |
| DHT/T† | Baseline | 0.05 ± 0.03 | 0.05 ± 0.02 | 0.05 ± 0.02 | 0.06 ± 0.04 |
| | Actual | 0.11 ± 0.04^a | 0.10 ± 0.04^a | 0.05 ± 0.03 | 0.06 ± 0.05 |

Values are expressed as means \pm 1 SD. †DHT/T = ratio of C_{avg} of DHT and T (nmol/liter units do not apply).

^a $P < 0.001$. Change from baseline significant vs. T patch.

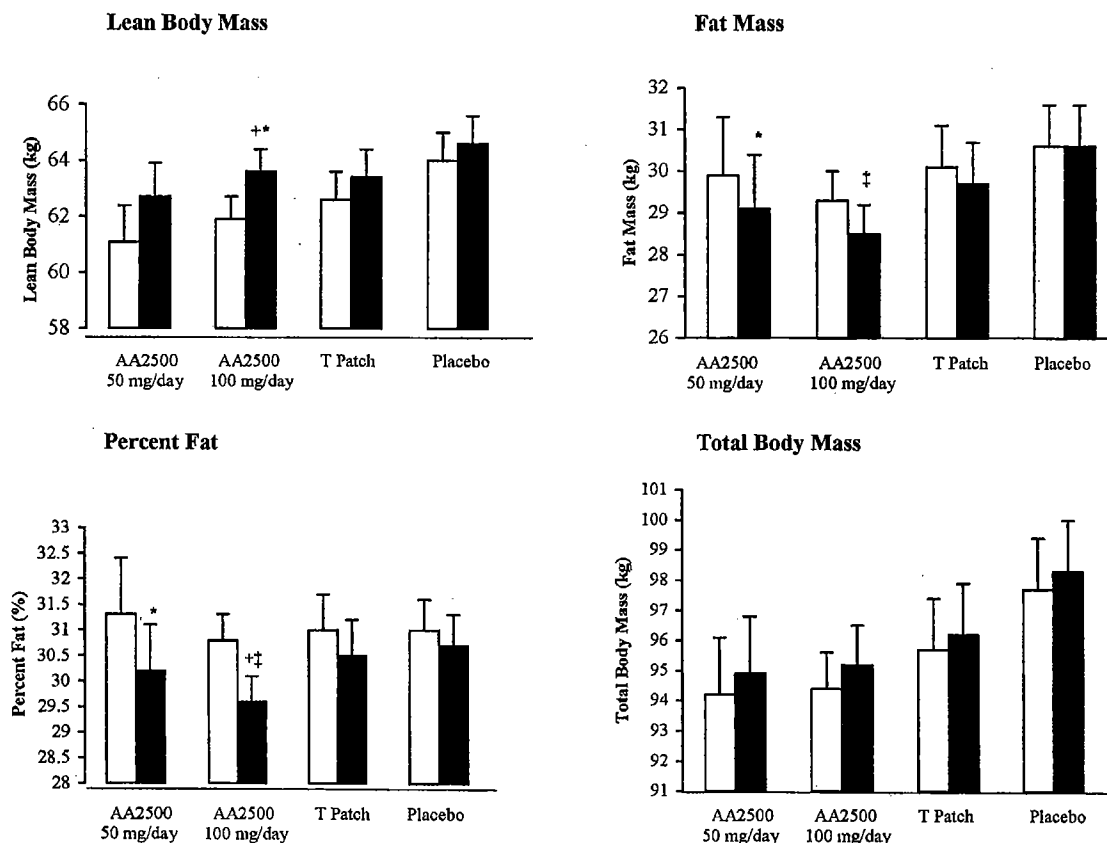


FIG. 3. Values are expressed as means \pm 1 SE in LBM, FM, %F, and TBM after treatment with 50 mg/d AA2500, 100 mg/d AA2500, T patch, and placebo. □, Baseline; ■, d 90; *, Significant vs. placebo: $P < 0.05$; +, significant vs. T patch: $P < 0.05$; ‡, significant vs. placebo: $P < 0.01$.

application site reactions, and irritation were observed much more frequently in the T patch group, compared with both AA2500 treatment groups and the placebo treatment group. Additionally, the T patches acted as an irritant in some patients who experienced classic signs of contact dermatitis,

whereas the AA2500 gel treatments resulted in minimal skin erythema in only a few patients with the incidence being similar to that observed in the placebo treatment group.

The 100 mg/d AA2500 treatment increased LBM to a greater degree than either the T patch or placebo, and both

TABLE 4. Sexual function scores: mean change from baseline to d 90

| | | AA2500 | | T patch | Placebo |
|---|----------|------------------------|--------------------------|------------------------|------------------------|
| | | 50 mg/d | 100 mg/d | | |
| Spontaneous erections (average weekly) | Baseline | 0.7 ± 0.9 | 0.8 ± 1.1 | 1.0 ± 1.3 | 1.0 ± 1.2 |
| | Change | 0.3 ± 1.3 | 0.7 ± 1.4 ^{e,f} | 0.3 ± 1.1 | 0.0 ± 1.0 |
| Motivation (average weekly) | Baseline | 1.6 ± 1.5 | 1.8 ± 1.4 | 1.6 ± 1.2 | 1.5 ± 1.2 |
| | Change | 0.2 ± 1.5 | 0.6 ± 1.4 ^{a,f} | 0.4 ± 1.1 ^d | 0.1 ± 1.2 |
| Desire (average daily) | Baseline | 2.3 ± 1.4 | 2.4 ± 1.4 | 2.2 ± 1.4 | 2.1 ± 1.4 |
| | Change | 0.5 ± 1.2 ^e | 1.0 ± 1.4 ^{b,f} | 0.6 ± 1.2 ^f | 0.5 ± 1.0 ^f |
| Performance (average weekly) | Baseline | 0.8 ± 0.9 | 0.8 ± 0.9 | 0.7 ± 0.8 | 0.8 ± 0.8 |
| | Change | 0.3 ± 1.1 | 0.5 ± 1.2 ^{a,f} | 0.3 ± 0.7 ^d | 0.2 ± 0.9 ^d |

Values are expressed as means ± 1 SD. Significant *vs.* placebo: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$. Significant within treatment group change from baseline: ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$.

TABLE 5. PSA: summary of changes to d 90 and classification of overall elevations

| | AA2500 | | T patch | Placebo |
|-----------------------------|------------------------|-----------|-----------|------------|
| | 50 mg/d | 100 mg/d | | |
| Baseline (ng/ml) | 1.2 ± 1.0 | 1.2 ± 0.9 | 1.4 ± 1.1 | 1.1 ± 1.0 |
| Change (ng/ml) | 0.3 ± 1.8 ^a | 0.1 ± 0.4 | 0.2 ± 0.6 | −0.1 ± 0.4 |
| PSA elevations ^b | | | | |
| >4.0 ng/dl | 1 | 4 | 6 | 3 |
| Transient | 1 | 2 | 2 | 1 |
| Persistent | 0 | 2 | 4 | 2 |

Baseline and change values are expressed as means ± 1 SD.

^a $P < 0.01$, significant *vs.* placebo.

^b Number of subjects experiencing at least one PSA value >4.

Skin Irritation Scores

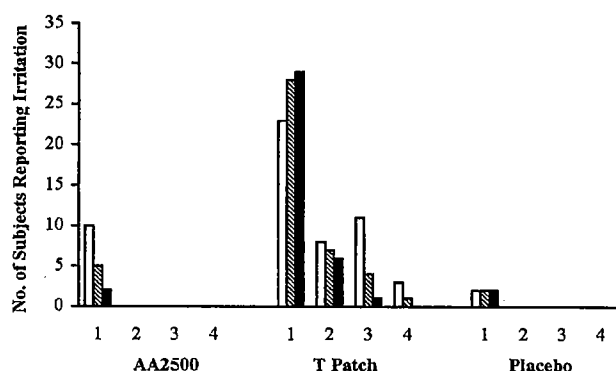


FIG. 4. Distribution of subjects with positive application site/skin irritation scores on d 30, 60, and 90. 1, Minimal erythema; 2, moderate erythema with sharply defined borders; 3, intense erythema with or without edema; 4, intense erythema with edema and blistering/erosion. The 50 mg/d and 100 mg/d AA2500 treatment groups combined. □, d 30; ▨, d 60; ■, d 90.

doses of AA2500 also resulted in a significantly greater decrease in FM and %F, compared with placebo. Normalization of serum T levels were achieved with both doses of AA2500 for average T levels and minimum T levels over a 24-h dosing period. In contrast, the T patch group was able to restore only average T levels. Previously reported data suggest that increases in LBM and decreases in FM are correlated with serum T levels (1). This difference in normalization may explain the greater increase in LBM and greater decrease in FM and %F observed with the AA2500 treatments. In previous studies in hypogonadal men, T replacement therapy has resulted in decreases in FM in some studies using injectable or transdermal T but not in other studies in which

either injectable or sublingual T has been administered. The difference in results observed in these previous reports might be due to lower serum T levels achieved by different T preparations.

Regarding DHT, although it is true that the AA2500 treatments produced higher serum levels at d 30 and 90, the DHT/T ratio remained stable and similar to that reported in normal men, demonstrating concordance with the naturally occurring 5 α -reductase conversion of T to DHT. The effect of serum DHT levels on the intraprostatic levels of DHT is not known. As with serum T levels, a prospective relationship between DHT serum levels and the incidence of prostate cancer has not been demonstrated (10, 11). Further long-term studies are needed to clarify the effect of increased DHT on the prostate.

There were no unexpectedly abnormal laboratory values and the incidence of clinically relevant abnormal findings was low. By d 90, patients in the AA2500 group who were administered 50 mg/d or 100 mg/d experienced statistically significant increases in hematocrit and hemoglobin, compared with patients receiving T patch or placebo. The increases observed in the T patch group were consistently greater than those observed in the placebo group but less than those observed in the AA2500 groups. This is likely a result of the lower serum T levels achieved with T patch. A previous study demonstrated similar increases in hematocrit and hemoglobin with T replacement therapy, with these increases being more marked with higher doses of T (1). These small increases in hematocrit and hemoglobin, which can occur with T replacement therapy, may even be beneficial in hypogonadal patients in whom anemia, lethargy, and fatigue are commonly found; however, a small percentage of treated individuals may increase their hematocrit levels to more than 55% and in turn be prone to the problems

associated with polycythemia. To this end, periodic monitoring of hematocrit is recommended to determine whether T therapy dose adjustments or termination (*i.e.* in the event hematocrit values do not fall below 55%) may be required.

Small increases in PSA similar to those seen in previous studies with T replacement therapy were observed in both AA2500 groups and in the T patch group. However, the magnitude of the increase in PSA, changes in I-PSS, and findings from DRE following treatment with either dose of AA2500 or T patch in this study were not of clinical concern. Although T has not been shown to induce cancer of the prostate, two patients on the T patch were diagnosed with prostate cancer during the study. This is not surprising because elderly men are at an increased risk of developing prostate cancer and the diagnosis can be made as a result of an elevated PSA subsequently leading to prostatic biopsy.

Very few adverse effects were reported following topical application of AA2500, and those that were reported were similar in type to the known class effects of T. Of particular note was the very low incidence of skin irritation reported with AA2500, which was comparable to placebo and significantly lower than T patch. Furthermore, no patient in the gel groups discontinued because of skin intolerance.

This study clearly shows that 100 mg/d AA2500 dose is superior to T patch in normalizing serum T and DHT in hypogonadal men. The AA2500 treatments resulted in increasing LBM (100 mg/d dose) and decreasing FM and %F to a greater degree than either the T patch or placebo. Furthermore, significant improvements from baseline and in comparison to placebo were observed for spontaneous erections, sexual motivation, sexual desire, and sexual performance with 100 mg/d AA2500 dose. Overall, this new, unique T gel (AA2500) can offer benefit over other transdermal preparations because of improved 24-h serum T levels and improved compliance as a result of a lower incidence of local dermal irritation.

Acknowledgments

We thank Drs. John Amory and William Bremner (University of Washington, Seattle, WA) for reviewing the manuscript and providing editorial guidance; Gary Nevins, D.C., for management of the clinical study; and Allen Secrest, B.Sc., for preparation of the manuscript.

Received July 8, 2002. Accepted March 5, 2003.

Address all correspondence and requests for reprints to: Christopher Steidle, M.D., Northeast Indiana Research, LLC, 2512 East DuPont Road, Suite 100, Fort Wayne, Indiana 46825. E-mail: chrisste@aol.com.

This work was supported by Auxilium Pharmaceuticals, Inc.

The North American AA2500 T Gel Study Group includes: M. Bas-tuba (San Diego Urology, La Mesa, CA); W. Borkon (Park Nicollet Clinic, St. Louis Park, MN); M. Borofsky (Clinical Research Center of Reading, West Reading, PA); P. Butler (Coast Urology Medical Group, Inc., La Jolla, CA); J. Caldwell (Radiant Research, Inc., Gainesville, FL); C. Cascione (Gainesville VAMC, Gainesville, FL); C. Corder (COR Clinical Research, Oklahoma City, OK); Z. Dalu (Radiant Research, Inc., St. Louis, MO); T. Decker (Strafford Medical Association, Dover, NH); L. Galitz (South Florida Bioavailability Clinic, Miami, FL); E. Gillie (ICSL

Clinical Studies, Fort Myers, FL); G. Gollapudi (Diabetes Center of the Southwest, Midland, TX); P. Hatcher (Volunteer Research Group, Knoxville, TN); K. Jacoby (Integrity Medical Research, Seattle, WA); M. Jayson (Medical & Clinical Research Associates, Bayshore, NY); W. Jones (Radiant Research, Inc., Boise, ID); L. Karsh (Western Urologic Research Center, Wheat Ridge, CO); J. Kaufman (Urology Research Options, Aurora, CO); W. Keating (SFM Clinical Trials, Scotland, PA); E. Killorin (Sandy Springs Urology, Atlanta, GA); T. Marbury (Orlando Clinical Research Center, Orlando, FL); J. McMurray (Medical Affiliated Research Center, Inc., Huntsville, AL); K. Meissner (Urology Consultants, San Antonio, TX); Y. Moy (The Connecticut Clinical Research Center/Urology Specialists, Waterbury, CT); T. Mulligan (McGuire VAMC Richmond, Richmond, VA); J. Newman (Quality Care Medical Center, Vista, CA); H. Offenbergl (Radiant Research, Inc., Daytona Beach, FL); M. Parker (Radiant Research, Inc., Tucson, AZ); R. Pearson (Urology Center, Memphis, TN); T. Phillips (Metrolina Medical Research, Charlotte, NC); W. Pittman (Urology Centers of Alabama, Homewood, AL); R. Rollins (Southeastern Urological Center, Tallahassee, FL); G. Salazar (Sun Research Institute, San Antonio, TX); S. Schwartz (Diabetes and Glandular Disease Research Associates, San Antonio, TX); R. Sievers (Wells Institute for Health Awareness, Kettering, OH); F. Snoy (Urology Group of New Mexico, Albuquerque, NM); J. Soufer (Phoenix Internal Medicine Associates, Waterbury, CT); C. Steidle (Northeast Indiana Research, Fort Wayne, IN); E. Stulberger (Physicians in Urology, Livingston, NJ); J. Susset (MultiMed Research, Providence, RI); M. Vance (University of Virginia Health System, Charlottesville, VA); J. Walton (Radiant Research, Inc., Greer, SC); W. Wells, Jr. (Alabama Research Center, Birmingham, AL).

References

1. Wang C, Swerdloff RS, Iranmanesh A, Dobs A, Snyder PJ, Cunningham G, Matsumoto M, Weber T, Berman N, Testosterone Gel Study Group 2000 Transdermal testosterone gel improves sexual function, mood, muscle strength, and body composition parameters in hypogonadal men. *J Clin Endocrinol Metab* 85:2839–2853
2. Arver S, Dobs AS, Meikle AW, Caramelli KE, Rajaram L, Sanders SW, Mazer NA 1997 Long-term efficacy, and safety of a permeation-enhanced testosterone transdermal system in hypogonadal men. *Clin Endocrinol (Oxf)* 47:727–737
3. Behre HM, von Eckardstein S, Kliersch S, Nieschlag E 1999 Long-term substitution therapy of hypogonadal men with transscrotal testosterone over 7–10 years. *Clin Endocrinol (Oxf)* 50:629–635
4. Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Holmes JH, Dlewati A, Staley J, Santanna J, Kapoor SC, Attie MF, Haddad Jr JG, Strom BL 1999 Effect of testosterone treatment on bone mineral density in men over 65 years of age. *J Clin Endocrinol Metab* 84:1966–1972
5. Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Lenrow DA, Holmes JH, Dlewati A, Santanna J, Rosen CJ, Strom BL 1999 Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. *J Clin Endocrinol Metab* 84:2647–2653
6. Jordan Jr WP, Atkinson LE, Lai C 1998 Comparison of the skin irritation potential of two testosterone transdermal systems: an investigational system and a marketed product. *Clin Ther* 20:80–87
7. Jordan Jr WP 1997 Allergy and topical irritation associated with transdermal testosterone administration: a comparison of scrotal and nonscrotal transdermal systems. *Am J Contact Dermatitis* 8:108–113
8. Marbury T, Hamill E, Bachand R, Sebre T, Smith T 2003 Evaluation of the pharmacokinetic profiles of the new testosterone topical gel formulation, Testim compared to AndroGel. *Biopharm Drug Dispos* 24:115–120
9. Dobs AS, Meikle AW, Arver S, Sanders SW, Caramelli KE, Mazer NA 1999 Pharmacokinetics, efficacy, and safety of a permeation-enhanced testosterone transdermal system in comparison with biweekly injections of testosterone enanthate for the treatment of hypogonadal men. *J Clin Endocrinol Metab* 84:3469–3478
10. Gann PH, Hennekens CH, Ma J, Longcope C, Stampfer MJ 1996 Prospective study of sex hormone levels and risk of prostate cancer. *J Natl Cancer Inst* 88:1118–1126
11. Mohr BA, Feldman HA, Kalish LA, Longcope C, McKinlay JB 2001 Are serum hormones associated with the risk of prostate cancer? Prospective results from the Massachusetts Male Aging Study. *Urology* 57:930–935

Detection of anabolic steroid administration: ratio of urinary testosterone to epitestosterone vs the ratio of urinary testosterone to luteinizing hormone

PAUL J. PERRY,^{1,3*} JOHN H. MACINDOE,² WILLIAM R. YATES,¹ SHANE D. SCOTT,³ and
TIMOTHY L. HOLMAN¹

Our goal in this study was to determine whether the urinary ratio of testosterone to luteinizing hormone (T/LH) as an indicator of exogenous anabolic steroid (AS) use is superior to the urinary ratio of testosterone to epitestosterone (T/E). After 2 weekly placebo injections, 19 subjects were given testosterone cypionate (TC) injections of 250 or 500 mg/week for 14 weeks followed by 14 weekly placebo injections. Patients were considered to have ceased taking TC if they tested negative 9 weeks after their last injection. For detection of illicit or supraphysiological TC (AS) use, the urinary T/E ratio of ≥ 6 yielded a false-negative rate of 46% and a false-positive rate of 4%. However, a urinary T/LH ratio of ≥ 30 produced a false-negative rate of only 24% and a false-positive rate of 13%. We conclude that the urinary T/LH ratio of ≥ 30 is a more sensitive marker of AS use than the urinary T/E ratio of ≥ 6 and remains sensitive for twice as long as urinary T/E.

INDEXING TERMS: abused drugs • sports medicine • GC-MS • androgens • anabolic steroids

The primary method for detecting illicit anabolic steroid (AS) use has been the analysis of urinary steroids.⁴ This methodology has been successful for the majority of steroids, especially the synthetic variety that have specific

structures that are easily identified by GC-MS. However, the detection and monitoring of anabolic compounds is not fail-safe. Detection of the illicit use of testosterone (T), a naturally occurring AS, has become a difficult clinical problem. Methods for detecting administration of exogenous T depend on distortions of the normal hormone profile in the user's urine [1]. Attempts to identify optimal markers of exogenous T administration from untimed urine samples in male athletes have uncovered several compounds as possible indicators of T abuse. In 1982, the ratio of androgen glucuronides to epitestosterone (E; 17 α -hydroxy-4-androsten-3-one) was adopted by the Medical Commission of the International Olympic Committee (IOC) in Los Angeles, with a cutoff point ≥ 6 being the sole test for illicit T self-administration [2, 3]; the expected urinary ratio of T/E among healthy subjects not using AS is ~ 1 [1]. However, analyses from all IOC-accredited laboratories in 1991 suggested that the majority of athletes who were using AS had switched from synthetic compounds to T pharmaceuticals to evade detection [4]. Consequently, covert AS use has become more difficult to detect.

The overall incidence of urinary T/E ≥ 6 in the general population of healthy males not abusing steroids is $<0.8\%$, as evaluated by Catlin and Hatton [5] and confirmed by Dehennin [4]. In general, the increase of the T/E ratio after high-dose T administration results from increased T excretion and a decrease of E output [6]. However, some athletes have produced false-positives, i.e., T/E ratios ≥ 6 with subsequent verification that no exogenous T had been administered [7]. Dehennin and Matsumoto [6] indicated that this problem could be reduced by taking into account the sulfate excretions of E (ES) in the ratio T/(ES + E), the relevant threshold being 2.85. Accordingly, Dehennin [4] suggested that using a

Departments of ¹ Psychiatry and ² Internal Medicine, College of Medicine, and ³ Department of Clinical Pharmacy, College of Pharmacy, University of Iowa, Iowa City, IA 52246

*Address correspondence to this author, at: S415 Pharmacy, University of Iowa, Iowa City, IA 52246. Fax 319-353-5646; e-mail paul-perry@uiowa.edu.

⁴ Nonstandard abbreviations: AS, anabolic steroid(s); T, testosterone; E, epitestosterone; IOC, International Olympics Committee; ES, epitestosterone sulfate; LH, luteinizing hormone; and TC, testosterone cypionate.

Received September 10, 1996; revised December 3, 1996; accepted December 20, 1996.

T/(ES + E) ratio of ≥ 3.0 would be a more sensitive marker of covert T use.

Dehennin [7] also noted that the joint misuse of T and E could also lead to false-negative test results, and the IOC in 1992 recommended that urinary E concentrations >150 $\mu\text{g/L}$ should be noted as abnormally high and therefore suspicious. False-negative results can also be produced by stimulation of testicular steroidogenesis by administering human chorionic gonadotropin, which would result in a concomitant increase in the urinary excretion rate of T and E but with no significant change in the T/E ratio [1]. Dehennin and Matsumoto [6] confirmed earlier reports of false negatives by demonstrating that, despite their determination that an average dose of 47 mg of exogenous testosterone per week would equal or exceed the IOC cutoff, 2 of 9 subjects receiving 72 mg of testosterone (100 mg of testosterone enanthate) per week for 6 months did not produce a T/E ratio ≥ 6 .

Dehennin [4] suggested that when a T/E ratio of 6 to 12 is found for the first time in subjects for whom no documentation of a previously normal ratio exists, some complimentary criteria should be examined. He found that the ratio of urinary T and E glucuronides to 5-androstene-3 β ,17 α -diol glucuronide was increased in the use of exogenous T and E use despite the T/E ratio being <6 . These findings indicate a need for further study of additional markers for detecting the administration of T.

Because the secretion of T is under the control of luteinizing hormone (LH), Brooks et al. [8] suggested that the urinary T/LH ratio might be a potentially useful marker for detecting administration of exogenous T. Kicman et al. [9] observed that high-dose T administration resulted in dose-dependent suppression of both serum and urinary LH. This was confirmed by Matsumoto [2], who found that the urinary LH excretion was reduced to a lesser extent than was the decrease in both E and T conjugates, such that the T/LH values were lower than those reported by Kicman et al.—also suggesting a need for more study.

Palonek et al. [3] reported significantly increased T/LH ratios in 11 healthy sedentary men participating in a WHO investigational program for male contraception. Each subject received 144 mg of T per week (200 mg of testosterone enanthate) for 9 months. The T/LH ratio increased from a mean of 0.052 (range 0.002–0.108) at baseline to 45.16 (1.28–252) at 3 months, 85.7 (8.3–238) at 6 months, and 71.7 (5.3–344) at 9 months. The authors indicated that, among all the different ratios or proposed markers they investigated, the urinary T/LH ratio showed the most dramatic increase (~ 1000 -fold). Of the other markers, the increase in the serum T/LH ratio was of similar magnitude as that of the urinary T/LH ratio, whereas the urinary T/E ratio had only a 50-fold increase. The investigators also reported that 1 of the 11 subjects produced a T/E ratio below the IOC cutoff at 3 and 9 months of administration and just over the threshold at 6 months. The T/LH ratio for the same subject was above

the upper reference limit at 3, 6, and 9 months. Palonek et al. concluded that increased serum and urinary T/LH ratios in the presence of a normal T/E ratio may indicate self-administration of both T and E.

Unanswered is whether the T/LH ratio might be more sensitive than the T/E ratio for identifying illicit use of AS. Thus the goal of the present study was to determine which laboratory test is most sensitive and specific for detecting the administration of exogenous T.

Materials and Methods

SUBJECTS AND STUDY DESIGN

Healthy male volunteers between ages 18 and 40 years were recruited and, after an explanation of the study, gave their signed informed consent. The study protocol was reviewed and approved by the Human Subjects Institutional Review Board and the Clinic Research Center of the University of Iowa. A standard drug history, developed by the National Institute on Drug Abuse, was administered before entry. Any subject who indicated he was currently using central nervous system stimulants other than modest amounts of caffeine (two cups of coffee per day) was excluded from the study.

Each subject received two weekly placebo doses of cottonseed oil, the vehicle for testosterone cypionate (TC). At the end of the 2-week placebo lead-in period, subjects were randomized to one of three doses of TC (100, 250, or 500 mg/week) given for 14 consecutive weeks. In our experience with AS users [10], the subjects' shortest average cycle was 7 weeks, the longest 14 weeks. Thus, we decided that subjects should be administered TC for a typical 14-week AS cycle to mimic the maximum cycling interval.

Subjects functioned as their own controls. They received weekly intramuscular injections of either TC or placebo (vehicle only) for 28 consecutive weeks. For the purpose of evaluating the effectiveness of the urine T/E ratio as an indicator of recent AS use, we considered only the subjects receiving supraphysiological TC doses (250 and 500 mg/week). A 100 mg/week dose is generally regarded as a physiological replacement dose in the majority of patients.

To monitor the subjects medically, we assessed their liver-function tests, fasting lipid profiles, thyroxine-binding globulin, sex-hormone-binding globulin, 24-h urinary free cortisols, serum free and total T, estradiol, LH, follicle-stimulating hormone, thyroid-stimulating hormone, and free thyroxine—obtained at baseline, at entry into the study, after the 2-week placebo injection period, and then biweekly for the remainder of the study. All endocrine samples were collected between 0700 and 0900 to minimize the chronotropic secretion effects of these hormones. Depo[®]-testosterone (TC), 200 g/L (200 mg/mL), was the proprietary product utilized for the study. The diluent (0.2 mL of benzyl benzoate, 9.45 mg of benzyl alcohol, and 560 mg of cottonseed oil per milliliter) was prepared by the Pharmaceutical Services Division of the

University of Iowa College of Pharmacy (an FDA-approved manufacturing group). At the end of the 14 weeks of TC administration, the subjects were switched to diluent-only injections.

ASSAYS

The urine drug screens were performed by Smith Kline Beecham Clinical Laboratories Sports Testing Center in Tucker, GA, a laboratory certified by the US Department of Health and Human Services. The initial drug screen and all subsequent screens were negative for AS (other than T), amphetamines, barbiturates, benzodiazepines, cocaine metabolites, methadone, methaqualone, opiates, phenylclidine, and propoxyphene.

Urine concentrations of T, E, LH, and creatinine were also determined in the samples (assayed by Smith Kline Beecham). The urine samples were refrigerated at 8 °C and were analyzed within 5–10 days after collection. If the T/E ratio was <6, the sample was discarded within 30 days. Urine drug screens were routinely obtained at weeks 0, 1, 4, 8, 12, 16 or 17, 20, 24, and 28; in some follow-up cases, they were obtained at weeks 40 and 92. AS screens and confirmations were performed by GC-MS on separate aliquots. Samples were initially screened for the substance abuse panel by Emit (Behring, Palo Alto, CA); all positive results were confirmed by GC-MS [11]. LH in urine was performed by Microparticle Enzyme Immunoassay with the Abbott Diagnostics (Chicago, IL) IMx system.

The T/E ratio was determined by GC-MS. Both free and conjugated T and E were extracted with C₁₈ solid-phase extraction columns (Bond Elute LRC; Varian, Harbor City, CA), hydrolyzed with β -glucuronidase (Boehringer Mannheim, Mannheim, Germany), and detected by monitoring characteristic ions with the mass spectrometer. Quantification and identification of T and E required selected-ion mode analysis in which the presumptive positive specimens were matched with the retention times and ion ratios of known compounds. The T calibration curve was linear between 2 and 400 μ g/L; that for E was linear between 2 and 500 μ g/L. The CV for the T/E ratio was 13.3%. The specificity of this method is extremely high: At the time of the performance of the assays, no compounds were known to interfere with either T or E.

The urine concentration of LH was determined with the IMx system kit for serum LH as described in the 1991 IMx LH package insert. To determine that there was no matrix effect for the assay, we added known amounts LH to urine and serum samples and found that the resulting calibration curves could be superimposed on each other and were linear between 2 and 600 IU/L. The lower limit of detection for this assay is 0.5 IU/L. The CV for the serum LH assay is 8.7% at 5.37 IU/L, 6.4% at 43.2 IU/L, and 6.2% at 82.5 IU/L.

All serum samples for determining free and total T were stored at -20 °C until assay. The T concentrations were quantified with Coat-A-Count® kits (Diagnostic

Products Corp., Los Angeles, CA) as described in the manufacturer's package insert (1995). The lower limits of detection were 40 ng/L for total T and 0.15 ng/L for free T. The inter- and intraassay CVs for the free T assay were 11.2% and 5.5%, respectively; those for total T were 10.4% and 8.8%, respectively.

Results

In all, 93 urine drug screen samples were obtained from the 19 subjects participating in the study who received supraphysiological doses of TC. Seven received 250 mg/week and 12 took 500 mg/week. None of the subjects was positive for exogenous AS use other than for the TC injections administered during weeks 2–15 of the study. Concentrations of free T in serum were analyzed 5–7 times between days 3 and 21 after the last TC injection for 17 of the 19 subjects. From these data, we calculated for each patient the terminal elimination rate (k_e) and the elimination half-life ($t_{1/2}$) for free T in serum. To determine k_e , we fit the T concentrations $f(t)$ and time points (t) to the following single exponential decay equation, where a is the concentration of T at time 0:

$$f(t) = ae^{-k_e t} \quad (1)$$

We determined $t_{1/2}$ as follows:

$$t_{1/2} = 0.693/k_e \quad (2)$$

A 21-day T sampling period was appropriate to determine the $t_{1/2}$ of exogenous T because gonadotropin-releasing-hormone stimulation tests indicated that the hypothalamic-pituitary-testicular axes of the subjects did not regain sufficient sensitivity to stimulate release of T until 4–6 weeks after discontinuation of the TC injections.

Table 1. Elimination half-life of free testosterone in 19 subjects.

| Subject | TC dose, mg/week | Free T elimination half-life, days |
|---------|------------------|------------------------------------|
| 2455 | 250 | 8.2 |
| 2908 | 250 | 6.0 |
| 3058 | 250 | 6.4 |
| 3626 | 250 | 4.8 |
| 5361 | 250 | 6.4 |
| 9298 | 250 | 6.4 |
| 0030 | 250 | 14.1 |
| 1078 | 500 | 6.0 |
| 2166 | 500 | 5.0 |
| 3415 | 500 | 7.3 |
| 4008 | 500 | 7.8 |
| 4045 | 500 | 4.9 |
| 4249 | 500 | 4.8 |
| 5340 | 500 | 7.4 |
| 6218 | 500 | Not available |
| 6534 | 500 | 6.8 |
| 9338 | 500 | 5.5 |
| 0153 | 500 | 4.2 |
| 0012 | 500 | Not available |

The individual elimination half-life data are presented in Table 1. There was no difference in half-life values between the weekly TC doses of 250 and 500 mg (Mann-Whitney $U = 25.0$, $P = 0.37$). The overall mean \pm SD elimination half-life for free T in serum after administration of TC was 6.6 ± 2.3 days. Based on these data, an 11-day $t_{1/2}$ would be 2 SD from the mean. Given that 97% of the exogenous T was excreted in 5 half-lives (i.e., 11-day half-life \times 5 half-lives = 55 days, or 8 weeks) and that pituitary sensitivity to gonadotropin-releasing hormone returned within 4–6 weeks of the last TC injection, subjects were assumed to have ceased taking ("be off") exogenous T by the time of the urine drug screen performed 9 weeks after the last TC injection.

The AS urine drug screen findings indicated that the urinary T/E ratio cutoff of ≥ 6 , the traditional laboratory marker to determine the use of exogenous T and used as such by the National Collegiate Athletic Association and the IOC, although quite specific for determining nonuse of T, is not a sensitive indicator for detecting illicit T usage. Table 2 illustrates this. Although the T/E ratio of >6 had 96% specificity in identifying our subjects as being off steroids by 9 weeks after their last dose, it was correct only 54% of the time for identifying our subjects as being on steroids during the 14 weeks of TC injections and in the 9 subsequent weeks when they received sham injections. As a practical matter, these data suggest that one of every two subjects using injectable TC will, both during injection periods and for 9 weeks afterwards, give a false-negative urine drug screen. Receiver operating characteristic (ROC) analysis of these data [12] identified a urinary T/E ratio of ≥ 1.2 as the cutoff value that provided optimum sensitivity and specificity for indicating use or nonuse of T. Resorting the data in Table 2 illustrates that use of a T/E ratio of ≥ 1.2 for a T-positive urine improves the sensitivity to 83% and the specificity decreases only somewhat, to 77%.

The potential usefulness of the urine T/LH ratio as an indicator of T use and nonuse is illustrated in Table 3. These data suggest that to maintain 100% specificity requires a threshold T/LH ratio of ≥ 74 , although the sensitivity at this cutoff is only 52%. However, by ROC analysis (data not shown), the urinary T/LH ratio cut-

Table 3. Contingency table for urinary T/LH ratios used as the threshold ratio for anabolic steroid use (TC 250 or 500 mg/week).

| T/LH ratio | No. of subjects | |
|-------------|-----------------|---------------------|
| | On TC | Off TC ^a |
| $\geq 74^b$ | 24 | 0 |
| $< 74^b$ | 22 | 47 |
| $\geq 30^c$ | 35 | 6 |
| $< 30^c$ | 11 | 41 |

^a Subjects who tested negative 9 weeks after their last TC injection.

^b $\chi^2 = 33.051$, $P < 0.0001$, sensitivity = 52%, specificity = 100%.

^c $\chi^2 = 37.814$, $P < 0.0001$, sensitivity = 76%, and specificity = 87%.

point that optimizes sensitivity and specificity is ≥ 30 . As Table 3 shows, use of a urinary T/LH ratio ≥ 30 increases sensitivity to 76% but decreases specificity to 87%.

Discussion

From a medical-legal standpoint the most worrisome finding of these data is the false-positive tests. Table 4 characterizes the false positives—i.e., a test result that is not negative at 9 weeks after the last TC injection—for the various testing schemes. Nine weeks is equivalent to the amount of time required to clear 97% of the exogenous TC. For the urinary T/E ratio of 6, only two subjects did not meet this criteria, whereas for the urine T/LH ratio of 30, four subjects did not meet this criteria. All six patients who tested "positive" were actually tested 9–25 weeks after their last TC injection. When contrasted with the half-life data, this suggests that the normalization of LH concentrations may lag behind the rate at which the exogenous T clears from the body. Moreover, in reality there are no truly false-positive test results. However, in no case did a subject's urine screen test positive before the start of the TC injections.

The mean \pm SD urinary T/LH and T/E ratios before the start of the TC injections were 3.8 ± 2.4 and 0.8 ± 1.3 , respectively. Other than when the subjects were receiving T injections, the only time there was a significant difference between pre-TC injection urinary T/E and T/LH ratios and the ratios after the start of the TC injections was 2 weeks after the last injection. For the urinary T/LH ratio, the mean difference between the baseline value and the ratio 2 weeks after the last injection was 29.8 ($t = 2.829$, $P < 0.02$, $df = 16$); for the urine T/E ratio, the mean difference was 14.9 ($t = 2.703$, $P < 0.02$, $df = 16$).

As suggested in Table 4, the urinary T/LH ratio of ≥ 30 is the screen most likely to detect AS use the longest, i.e., as long as 25 weeks after the last injection. Using the urinary T/LH ratio ≥ 30 as a marker showed that 4 of 19 (21%) subjects tested positive 9–25 weeks after their last injection of T. In contrast, use of the urinary T/E ratio ≥ 6 found only 2 of 19 (11%) patients positive for steroid usage at 9 weeks after their last T injection. Fig. 1 chronologically contrasts the mean urine T/LH and T/E ratios. Inspection of Fig. 1 suggests that the urinary T/LH

Table 2. Contingency table for various urinary T/E ratios used as the threshold ratio for anabolic steroid use (TC 250 or 500 mg/week).

| T/E ratio | No. of subjects | |
|--------------|-----------------|---------------------|
| | On TC | Off TC ^a |
| $\geq 6^b$ | 25 | 2 |
| $< 6^b$ | 21 | 45 |
| $\geq 1.2^c$ | 38 | 11 |
| $< 1.2^c$ | 8 | 36 |

^a Subjects who tested negative 9 weeks after their last TC injection.

^b $\chi^2 = 28.312$, $P < 0.0001$, sensitivity = 54%, specificity = 96%.

^c $\chi^2 = 32.689$, $P < 0.0001$, sensitivity = 83%, specificity = 77%.

Table 4. False-positive rates for use of supraphysiological doses of anabolic steroids in different urine testing schemes.

| Test cutoff | False-positive rates | | Subject description | | |
|----------------|----------------------|-----------------|---------------------|------------------------------|----------------------|
| | % | No. of subjects | TC dose, mg/week | No. of weeks since last inj. | Ratio |
| T/E ≥ 6 | 4 | 2 | 500 | 9 | 7.3 |
| | | | 500 | 9 | 6.5 |
| T/LH ≥ 30 | 13 | 4 | 500 | 9 | 73 |
| | | | 500 | 9 & 13 ^a | 65 & 35 ^a |
| | | | 250 | 13 | 64 |
| | | | 250 | 13 & 25 ^a | 47 & 34 ^a |

^a Same subject, two times.

ratio returns to baseline at a slower rate than the urinary T/E ratio does, thereby explaining the greater number of false-positive results for T/LH in this group. To prove this point, we regressed the mean T/E and T/LH ratios against their timepoints at weeks 17, 20, 24, 28, and 40 and fit this as a monoexponential decay curve. The regression line intersects the critical T/LH ratio of 30 at 7.9 weeks after the last TC injection (T/LH ratio = $90.2 e^{-0.14(\text{week})}$, $r^2 = 0.86$). However, the T/E ratio fitted to the exponential equation (T/E ratio = $12.3 e^{-0.1916(\text{week})}$, $r^2 = 0.80$) intersects the ratio of 6 at 3.7 weeks. Both models, therefore, demonstrate why more subjects test positive for a longer time when assessed with the T/LH ratio.

It is not uncommon for nonpower athletes (e.g., distance runners, swimmers, tennis players, soccer players) to utilize physiological doses of T (i.e., TC 100 mg/week) to counter the catabolic effects of stress and exercise on muscle. We measured urine T/E and urine T/LH in seven subjects who were administered TC at 100 mg/week. Monoexponential regression equations for the T/E and T/LH ratios to return to baseline values were based on the mean ratios measured in these subjects at weeks 17, 20, and 24 after cessation of TC injections. The urinary T/E ratio, when fitted as an exponential decay equation

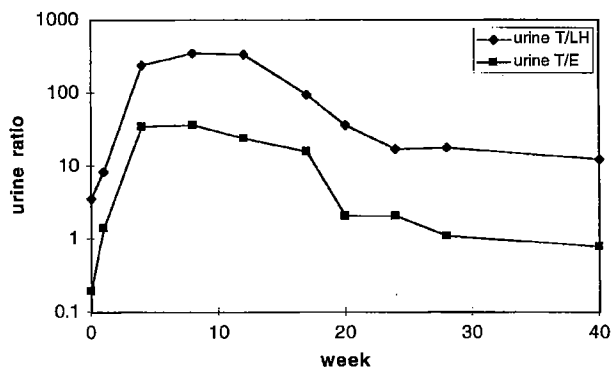


Fig. 1. Urine T/LH ratios and T/E ratios in 19 subjects receiving TC, 250 or 500 mg/week.

(T/E ratio = $8.3 e^{-0.2072(\text{week})}$, $r^2 = 0.82$), intersects the ratio of 6 at 1.6 weeks, whereas the urinary T/LH ratio, fitted to the equation T/LH ratio = $48.9 e^{-0.2148(\text{week})}$ ($r^2 = 0.99$), intersects the ratio of 30 at 2.3 weeks. These data suggest that is debatable whether TC at 100 mg/week is actually a physiological replacement dose: Some athletes may test positive even at this small a dose of T.

In conclusion, we find that the urinary T/LH ratio is a more sensitive and specific test for a longer time for investigating AS use than is the urinary T/E ratio. Supporting this finding is the fact that, unlike the case for E, there are no commercially available FDA-approved LH products. This advantage alone makes the urinary T/LH ratio a considerably more practical screening test than the urinary T/E ratio.

This project was supported by a grant from the National Institute on Drug Abuse at the National Institutes of Health (RO1DA08347).

References

- Kicman A, Oftebro H, Walker C, Norman N, Cowan DA. Potential use of ketoconazole in a dynamic endocrine test to differentiate between biological outliers and testosterone use by athletes. *Clin Chem* 1993;39:1798-803.
- Matsumoto AM. Effects of chronic testosterone administration in normal men: safety and efficacy of high-dosage testosterone and parallel dose-dependent suppression of luteinizing hormone, follicle-stimulating hormone, and sperm production. *J Clin Endocrinol Metab* 1990;70:282-7.
- Palonek E, Gottlieb C, Garle M, Björkhem I, Carlstrom K. Serum and urinary markers of exogenous testosterone administration. *J Steroid Biochem Mol Biol* 1995;55:121-7.
- Dehennin L. Detection of simultaneous self-administration of testosterone and epitestosterone in healthy men. *Clin Chem* 1994;40:106-9.
- Catlin DH, Hatton CK. Use and abuse of anabolic and other drugs for athletic enhancement. *Adv Intern Med* 1991;36:399-424.
- Dehennin L, Matsumoto A. Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport. *J Steroid Biochem Mol Biol* 1993;44:179-89.
- Dehennin L. On the origin of physiologically high ratios of urinary testosterone to epitestosterone: consequences for reliable detection of testosterone administration by male athletes. *J Endocrinol* 1994;142:353-60.
- Brooks R, Jeremiah G, Webb W, Wheeler M. Detection of anabolic steroid administration to athletes. *J Steroid Biochem* 1979;11:913-7.
- Kicman A, Brooks R, Collyer S, Cowan DA, Nanjee MN, Southan GJ, Wheeler MJ. Criteria to indicate testosterone administration. *Br J Sports Med* 1990;24:253-64.
- Perry PJ, Andersen KH, Yates WR. Illicit anabolic steroid use in athletes: a case series analysis. *Am J Sports Med* 1990;18:422-8.
- Sample RHB, Baenziger JC. Gas chromatography/mass spectrometric detection of anabolic steroids. In: Deutsch J, ed. *Analytical aspects of toxicology*. New York: JR Wiley and Sons, 1989:247-72.
- Somoza E, Mossman D. Neuropsychiatric decision making: designing nonbinary diagnostic tests. *J Neuropsychiatry Clin Neurosci* 1991;3:197-200.

A Single Dose of the Potent Gonadotropin-Releasing Hormone Antagonist Acyline Suppresses Gonadotropins and Testosterone for 2 Weeks in Healthy Young Men

KAREN L. HERBST, ANDREA D. COVIELLO, STEPHANIE PAGE, JOHN K. AMORY, BRADLEY D. ANAWALT, AND WILLIAM J. BREMNER

Department of Medicine, Division of Endocrinology, Charles R. Drew University (K.L.H.), Los Angeles, California 90059; Center for Research in Reproduction and Contraception, Department of Medicine, University of Washington (A.D.C., S.P., J.K.A., B.D.A., W.J.B.), and Medical Service, Department of Veteran Affairs, Puget Sound Health Care System (B.D.A.), Seattle, Washington 98195

Acyline is a novel GnRH antagonist that reliably inhibits gonadotropins and testosterone (T) levels in men for 48 h after a single dose up to 75 $\mu\text{g/kg}$. In this study we examined gonadotropin and T levels in 28 healthy young men administered acyline as single doses of 150 or 300 $\mu\text{g/kg}$ or serial injections of 75 $\mu\text{g/kg}$. A single 300 $\mu\text{g/kg}$ dose of acyline suppressed gonadotropins and T to castrate levels for 15 d (baseline, 21.1 ± 3.1 ; nadir, 1.95 ± 0.4 nmol/liter; mean \pm SEM; $P < 0.05$). Serum acyline levels peaked 90 min after the injection of 300 $\mu\text{g/kg}$ acyline to a maximum concentration of 112.4 ± 18 ng/ml ($n = 7$; $t_{1/2} = 4.9$ d). Injections of 75 $\mu\text{g/kg}$ acyline every 2 d for five

doses suppressed gonadotropins for more than 20 d (nadir T, 1.06 ± 0.17 nmol/liter; $P < 0.05$ compared with baseline). Adverse events were mild and included erythema and pruritus at the injection site. Acyline, therefore, is one of the most potent peptide GnRH antagonists studied to date with minimal adverse events. A twice monthly injection of acyline could be used as a potent suppressor of the GnRH axis to advance the development of a hormonal male contraceptive or for treatment of hormonally dependent disease. (*J Clin Endocrinol Metab* 89: 5959–5965, 2004)

GnRH is a hypothalamic decapeptide that is synthesized and released in a pulsatile manner from hypothalamic neurosecretory cells and regulates the synthesis and release of pituitary gonadotropins that, in turn, regulate steroidogenic and gametogenic functions of the gonads. GnRH plays a rate-limiting role in reproductive processes, and thousands of GnRH analogs have been generated because of their potential clinical utility. Most GnRH analogs are employed in the treatment of prostate cancer and for their utility in the disruption of LH surges for the regulation of ovulation induction in women undergoing *in vitro* fertilization (1–3). Other clinical uses for GnRH analogs include endometriosis and other gynecological diseases (4, 5), precocious puberty (6), and hormonal male contraception (7, 8). Both GnRH agonists and antagonists suppress gonadotropins and gonadal steroids, but the administration of GnRH agonists is accompanied by an initial gonadotropin and gonadal hormone surge known as a flare, delaying suppression by 7–14 d (2). GnRH antagonists do not cause flare, because they competitively block and inhibit GnRH-induced GnRH receptor gene expression, leading to immediate pituitary suppression.

Acyline is a GnRH antagonist sponsored by the NICHD that reliably maintained suppression of gonadotropins and testosterone (T) for 48 h with doses up to 75 $\mu\text{g/kg}$ by sc injection (9). We sought to determine whether larger or serial

doses of acyline would suppress gonadotropins and T for sufficiently long periods of time to allow a depot formation of acyline to be conveniently used for clinical applications such as a male hormonal contraception.

We administered acyline as single doses of 150 and 300 $\mu\text{g/kg}$ and as serial injections of 75 $\mu\text{g/kg}$ and monitored serum gonadotropin and T levels for a period of 30 d or until serum gonadotropins and T returned to baseline levels.

Subjects and Methods

Acyline

Acyline was originally synthesized by Jean Rivier at The Salk Institute (La Jolla, CA) (10) and is distributed by the NICHD. Acyline is prepared as a lyophilized powder (4.4 mg/vial) and is stored at -20°C . Acyline-lyophilized powder was suspended in bacteriostatic water to a final concentration of 2 mg/ml for injection into sc abdominal tissue. Serum levels of acyline were measured by RIA in a subset of subjects after the injection of 300 $\mu\text{g/kg}$ acyline, using a specific antiserum and a proprietary peptide with authentic peptide standard (Woods Assay, Inc., Portland, OR) as described previously (11). The sensitivity of the assay for acyline was 0.35 ng/ml.

Subjects

All study procedures involving human subjects were approved by the institutional review board at University of Washington and were performed at the University of Washington Clinical Research Center in accordance with institutional guidelines. Thirty-five men (age, 18–50 yr) were recruited by flyers posted on local college campus bulletin boards. All subjects were healthy, eugonadal men with normal medical histories and baseline physical examinations, including normal testicular size by Prader orchidometer and prostate size by digital rectal exam, serum chemistries, complete blood count, and gonadotropin and T levels. We excluded subjects who were current smokers, drank more than 7 oz

Abbreviations: ACY 150, 150 $\mu\text{g/kg}$ Acyline; T, testosterone.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

alcohol weekly, or had taken prescription medications or any sex steroid hormone within the last 6 months. Of these men, 34 were screened for study eligibility. Six men were excluded or withdrawn from the study after the screening process and before drug treatment: one man had low T levels, one man had abnormal hematology results at screening, one man had an elevated alanine aminotransferase test value, and two men had time constraints that prevented study participation. One subject was screened but did not begin the drug treatment phase, because recruitment for the study was complete.

Subjects were assigned to one of four groups ($n = 7$): group 1, single dose of 150 $\mu\text{g/kg}$ acyline on d 0 (ACY 150); group 2, single dose of 300 $\mu\text{g/kg}$ acyline on d 0 (ACY 300); group 3, 75 $\mu\text{g/kg}$ acyline injections on d 0, 4, and 8 and placebo on d 2 and 6 (ACY 75 \times 3); and group 4, 75 $\mu\text{g/kg}$ acyline injections on d 0, 2, 4, 6, and 8 (ACY 75 \times 5).

Acyline was administered by sc injection in the abdomen between 0700 and 1000 h. For ACY 150 and 300 groups, blood samples were obtained at 30, 60, 90, and 120 min; 3, 4, 6, 8, 12, and 24 h; and 2, 3, 4, 7, 9, 11, 13, 15, 17, 19, 21, 25, and 30 d after injection. For the serial injection groups ACY 75 \times 3 and 75 \times 5, blood samples were obtained on d 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 21, 25 and 30. All research subjects were monitored for 30 d with vital signs determined, and laboratory tests and physical exams performed.

Measurements

Hormones. FSH, LH, and T levels were measured by immunofluorometric assay (Delfia, Wallac Oy, Turku, Finland). Samples from a given individual were measured in a single assay. The sensitivities of the assay for FSH and LH were 0.016 and 0.019 IU/liter, respectively. For low, mid, and high pooled values of 0.054, 1.04, and 20.8 IU/liter FSH, the intraassay coefficients of variation were 12%, 1.9%, and 2.9%, and the interassay coefficients of variation were 18%, 6.1%, and 4.1%, respectively. For low, mid, and high pooled values of 0.056, 0.95, and 15.6 IU/liter LH, the intraassay coefficients of variation were 6.5%, 3.9%, and 5.4%, and the interassay coefficients of variation were 21%, 8%, and 6.6%, respectively. The assay sensitivity for T was 0.5 nmol/liter. For low, mid, and high pooled values of 3.8, 10.6, and 24.4 nmol/liter T, the intraassay coefficients of variation were 9.6%, 5.2%, and 6.1%, and the interassay coefficients of variation were 12%, 8.2%, and 6.7%, respectively. If serum T levels had not returned to baseline within 30 d after the injection of acyline, subjects were requested to return for additional blood sampling until serum T levels had normalized.

Serum laboratory tests. Screening and monitoring laboratory tests for complete blood count, electrolytes, glucose (chemistry 7), calcium, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, bilirubin, and total protein were performed at the University of Washington.

Statistics

FSH, LH, and T are expressed as the mean hormone level \pm SEM. For statistical analysis, all hormone data were log-transformed and then back-transformed for ease of presentation. Comparisons of data within groups and between groups were analyzed with ANOVA (SigmaStat, SPSS, Inc., Chicago, IL). Evidence for change from baseline within a group was further analyzed at each time point by paired *t* tests. The area under the curve was calculated using the trapezoid rule from time zero to the last measured level without smoothing or curve fitting (PK Solutions, Summit Research, Montrose, CO). The half-life ($t_{1/2}$) was calculated from 3 d to the last measured level. For all comparisons, an α of 0.05 was considered significant.

Results

Study population

The 28 subjects enrolled in this study were eugonadal and lean by body mass index (Table 1). There were no significant differences in baseline parameters between groups.

Acyline administration

Acyline was initially suspended in bacteriostatic water at a concentration of 8.8 mg/ml, which allowed acyline to be administered as a single, small volume injection at a concentration of 300 $\mu\text{g/kg}$ (2.4-ml injection for a 70-kg man). Nodule formation at the sites of acyline injection (lingering for as long as 30 d postinjection) suggested that the drug might be forming a gel in the fat tissue in a volume large enough to be palpable, similar to other GnRH antagonists (12). Suppression of gonadotropins and T was also inconsistent (data not shown). Acyline was therefore administered as a 2.0 mg/ml solution in water that does not lead to palpable nodule formation when injected into sc tissue (9), but did require multiple injections for a dose of 150 or 300 $\mu\text{g/kg}$. A 70-kg man administered 300 $\mu\text{g/kg}$ acyline at a concentration of 2.0 mg/ml would have an sc injection of 10.5 ml in four or five divided doses (2–3 ml/injection).

Single injections of acyline

Gonadotropins. Baseline levels of FSH and LH are shown in Table 1. Both FSH and LH decreased rapidly after a single dose of 150 or 300 $\mu\text{g/kg}$ acyline in all subjects (Fig. 1). In the ACY 150 group, FSH levels dropped significantly below baseline 8 h after injection, reaching a nadir at 3 d (1.06 ± 0.35 IU/liter), and remained significantly below baseline for 4 d after injection. LH levels decreased significantly below baseline by 1.5 h after injection, reached a nadir at 2 d (0.27 ± 0.05 IU/liter), and remained significantly below baseline for 3 d after injection.

In the ACY 300 group, FSH levels decreased significantly below baseline 1 h after injection and remained at these levels for 21 d, reaching a nadir on d 11 (0.26 ± 0.04 IU/liter). LH levels decreased significantly below baseline by 1.5 h after injection and reached a nadir at 2 d (0.17 ± 0.1 IU/liter), remaining significantly below baseline levels for 15 d. Gonadotropin levels tended to rebound above baseline by d 30 after the injection of acyline, but there was no significant difference between baseline and d 30 FSH or LH levels in the ACY 150 or ACY 300 groups.

T. Baseline levels of T for ACY 150 and ACY 300 are shown in Table 1. T rapidly decreased significantly below baseline

TABLE 1. Baseline parameters of subjects

| Group | ACY 150 | ACY 300 | ACY 75 \times 3 | ACY 75 \times 5 |
|--------------------------------------|----------------|----------------|-------------------|-------------------|
| n | 7 | 7 | 7 | 7 |
| Age (yr) | 26.6 \pm 2.7 | 31.9 \pm 3.8 | 30.9 \pm 2.1 | 23.0 \pm 1.8 |
| Body mass index (kg/m ²) | 26.1 \pm 1.7 | 24.5 \pm 0.7 | 25.6 \pm 1.1 | 26.2 \pm 1.6 |
| FSH (IU/liter) | 2.5 \pm 0.3 | 2.7 \pm 0.8 | 3.1 \pm 0.5 | 2.0 \pm 0.3 |
| LH (IU/liter) | 3.2 \pm 0.5 | 3.3 \pm 0.7 | 3.5 \pm 0.2 | 4.0 \pm 0.4 |
| T (nmol/liter) | 21.1 \pm 3.1 | 21.6 \pm 4.2 | 20.0 \pm 2.6 | 26.1 \pm 3.3 |

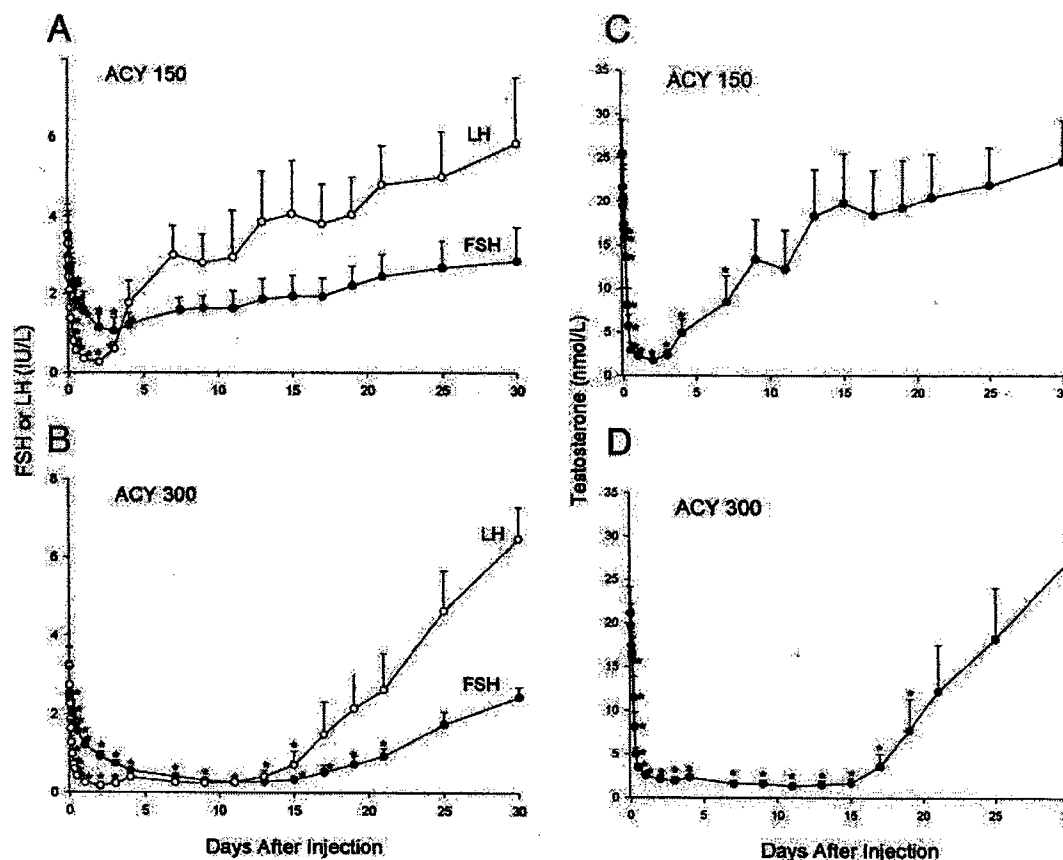


FIG. 1. Single injections of acyline rapidly suppress gonadotropins and T. Serum values of FSH (●) and LH (○) after 150 (A) and 300 (B) µg/kg acyline and serum values of T (●) after 150 (C) and 300 (D) µg/kg acyline are shown. Time is expressed as days after the first injection of acyline. Values (n = 7) are expressed as the mean ± SEM. *, $P < 0.05$ vs. baseline.

2 h after injection in all subjects after single acyline doses and decreased below castrate levels (5 nmol/liter) by 12 h after injection (Fig. 1). T levels reached a nadir in the ACY 150 group 2 d after injection (1.75 ± 0.28 nmol/liter) and began to rise but remained significantly below baseline through d 7 after injection. One subject's T level remained below baseline levels until d 37.

T levels reached a nadir on d 2 after injection in the ACY 300 group (1.95 ± 0.38 nmol/liter) and remained at approximately this same level through d 15. T levels began increasing on d 17 after injection, remaining significantly below baseline levels until d 19 after injection, but were not significantly different from baseline levels on d 21. One subject's T level remained below baseline levels until d 35. On the average, there was no significant difference between baseline and d 30 T levels in either group.

Multiple injections of acyline

Gonadotropins. In the ACY 75X3 group, FSH and LH levels decreased significantly below baseline on d 2 (Fig. 2, A and B). Gonadotropin levels increased before the next injection on d 4, but remained significantly below baseline, then decreased on d 6, reaching steady suppressed levels on d 10–18 after the third injection. The nadir level of FSH on d 18 was 0.39 ± 0.08 IU/liter, and the nadir level for LH was 0.16 ± 0.04 IU/liter on d 10. LH and FSH levels remained signifi-

cantly below baseline through d 21 and 25, respectively, but the levels normalized and were not significantly different from baseline levels by d 30.

In the ACY 75X5 group, LH and FSH decreased significantly below baseline by d 2 (Fig. 2, C and D). LH levels remained significantly suppressed below baseline from d 2–16, with a nadir level on d 10 (0.07 ± 0.01 IU/liter). FSH levels gradually decreased to a nadir on d 12 (0.1 ± 0.01 IU/liter) and remained significantly below baseline on d 2–25.

T. In the ACY 75X3 group, T decreased significantly below baseline levels after the first acyline injection (Fig. 3A), with small rebound increases before the second and third injections. T levels reached a nadir on d 10 after the third injection (1.29 ± 0.22 nmol/liter), remained significantly below baseline levels from d 2–21, and returned to baseline by d 30. Two subjects' T levels remained below baseline on d 30, but returned to baseline levels by d 33 and 47, respectively.

In the ACY 75X5 group, T levels decreased rapidly and significantly below baseline, reaching a nadir on d 17 (1.06 ± 0.17 nmol/liter) after the first injection and remained significantly suppressed through d 25, returning to baseline levels by d 30. Four subjects' T levels remained significantly below baseline levels on d 30, returning to baseline levels on d 36, 37, 45, and 47, respectively.

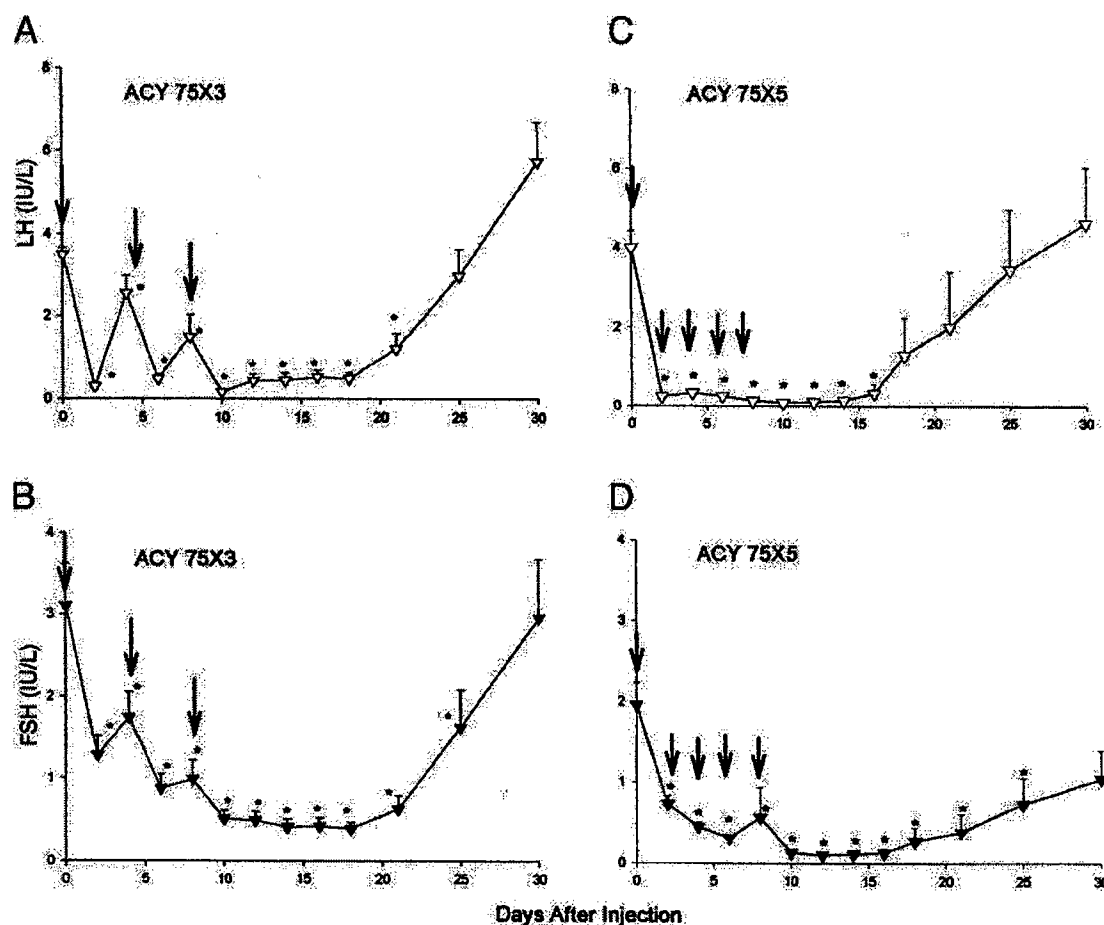


FIG. 2. Multiple injections of 75 µg/kg acyline can maintain suppression of gonadotropins for more than 20 d. Serum values of LH (A; Δ) and FSH (B; \blacktriangle) after three injections of 75 µg/kg acyline spaced 4 d apart (arrows) and LH (C) and FSH (D) levels after five injections of 75 µg/kg acyline spaced 2 d apart (arrows) are shown. Time is expressed as days after the first injection of acyline. Values ($n = 7$) are expressed as the mean \pm SEM. *, $P < 0.05$ vs. baseline.

Acyline pharmacokinetics

Serum acyline levels were measured in all seven subjects in the ACY 300 group (Fig. 4). Acyline reached a maximum concentration of 112.4 ± 6.9 ng/ml 90 min after injection and remained significantly elevated above background through d 30. Blood levels of acyline dropped by 50% at 4.9 d.

Adverse events and safety

Skin reactions were the most common side-effect noted after sc acyline injections. A mild pink blush occurred at the site of injection in 22 of 28 subjects (78.5%), lasting up to 120 min. The blush occurred in 94% of injections in subjects who experienced it. Pruritus at the site of injection occurred in 75% of subjects, with an average score of 2.3 ± 0.1 arbitrary units of 5: 0 = no itch, 1 = barely noticeable, 2 = mild, 3 = moderate, 4 = severe, and five = the worst itch ever experienced. The pruritus persisted for approximately 40 min on the average. Nodules or deep induration were noted in three individuals, one in each of the groups receiving single acyline doses (although multiple injections), and persisted at 2 and 11 d; in one subject receiving serial injections, they persisted for 2 d. Bruising at the site of injection was also noted in 12

individuals (42.9%), although not at all injection sites. During the hypogonadal period (T, <5 nmol/liter), 10 subjects experienced decreased libido or fatigue (35.7%), and two experienced hot flashes or significant changes in mood/irritability (7.1%). One subject experienced myalgia without signs of infection, including fever. There were no significant changes in any chemistry parameter during the study, including aspartate aminotransferase and alanine aminotransferase. The hematocrit decreased slightly in all 28 subjects from an average baseline value of $42.5 \pm 0.46\%$ to $41.1 \pm 0.49\%$ ($P < 0.01$), remaining in the normal range (38–50%). The white blood cell and platelet counts were unaffected by acyline administration.

Discussion

GnRH antagonists competitively block and inhibit GnRH-induced GnRH receptor gene expression, leading to immediate pituitary suppression (13) without the surge in gonadotropins and T seen after GnRH agonist administration (14). The GnRH antagonist, acyline, was developed to have greater potency and less histamine-mediated skin irritation than previous antagonists (10). A single injection of 300

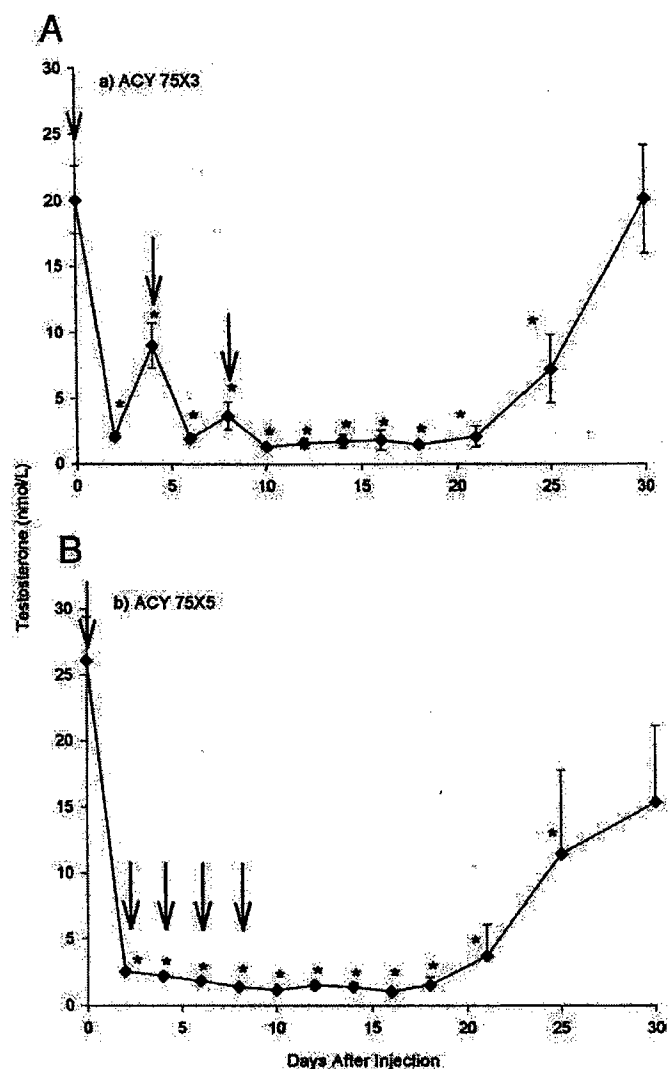


FIG. 3. Multiple injections of 75 $\mu\text{g/kg}$ acyline can maintain suppression of T for more than 20 d. Serum values of T (\diamond) after three injections of 75 $\mu\text{g/kg}$ acyline spaced 4 d apart (A, arrows) and five injections of 75 $\mu\text{g/kg}$ acyline spaced 2 d apart (B, arrows). Time is expressed as days after the first injection of acyline. Values ($n = 7$) are expressed as the mean \pm SEM. *, $P < 0.05$ vs. baseline.

$\mu\text{g/kg}$ acyline in this study rapidly and significantly suppressed gonadotropins and T to castrate levels (T, < 5 nmol/liter) for 15 d. Five injections of 75 $\mu\text{g/kg}$ acyline spaced 2 d apart also immediately suppressed gonadotropins and T and maintained T within the castrate range for up to 21 d. Five injections of 75 $\mu\text{g/kg}$ acyline might have suppressed the hypothalamic-pituitary-gonadal axis longer than the single 300 $\mu\text{g/kg}$ dose, because the cumulative dosage administered was greater at 375 $\mu\text{g/kg}$. Alternatively, because 75 $\mu\text{g/kg}$ is known to rapidly and effectively suppress gonadotropins and T for up to 48 h (9), serial injections of acyline every 2 d over an 8-d period might simply maintain that 48-h suppression. However, if the latter were true, then gonadotropins would be expected to begin returning to baseline approximately 48 h after the last injection. Instead, FSH and LH were still suppressed 8 d after the last of five injections of 75 $\mu\text{g/kg}$ acyline.

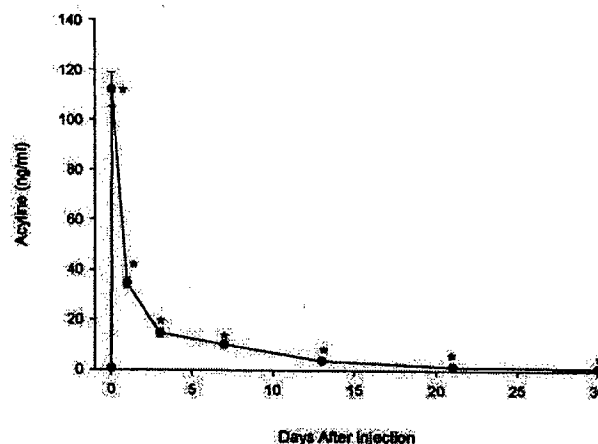


FIG. 4. Serum levels of acyline (nanograms per milliliter) remained significantly elevated for 30 d after sc injection of 300 $\mu\text{g/kg}$ acyline in seven healthy young men. Values are the mean \pm SEM after subtraction of 1 ng/ml background levels. *, $P < 0.05$ vs. baseline.

Cetrorelix is a GnRH antagonist that has similar potency to acyline (9, 15). Data examining the effect of cetrorelix on pituitary GnRH receptor expression and localization might help explain why serial injections of 75 $\mu\text{g/kg}$ acyline induce longer suppression of gonadotropins than a single 300 $\mu\text{g/kg}$ injection (15–18). GnRH receptors were significantly down-regulated for at least 72 h in rat pituitaries, accompanied by suppression of serum LH and T, after a single injection of 100 μg cetrorelix (16). The lowest receptor level was found 6 h after the injection of cetrorelix, but a marked recovery in receptor number was observed at 48 h. A major decrease in the expression of mRNA for pituitary LH-releasing hormone receptors was also found after chronic treatment with cetrorelix (17). This suppression is not believed to be a direct effect on gene expression of the GnRH receptor, but due to the fact that GnRH antagonists prevent up-regulation of receptor mRNA expression induced by GnRH (18). These data suggest that the prolonged suppression of gonadotropins and T by serial injections of 75 $\mu\text{g/kg}$ acyline is not only a reflection of the concentration of the antagonist at the level of the receptor, but that administration every 48 h might have prevented the up-regulation of GnRH receptor mRNA expression longer than the single dose of 300 $\mu\text{g/kg}$.

The question then becomes how is acyline best administered. Subjects and patients would probably prefer a single dose of sc acyline twice a month to serial injections. However, serial injections maintained suppression of gonadotropin and T levels longer than either single dose, as demonstrated by the four subjects whose T levels remained significantly below baseline more than 30 d after the start of the five injections. A minimal dose for a twice monthly injection might be 225 $\mu\text{g/kg}$, because gonadotropins and T were suppressed in the hypogonadal range for 14 d after three injections of 75 $\mu\text{g/kg}$ acyline. Alternatively, a higher dose injection than 300 $\mu\text{g/kg}$ might maintain acyline concentrations in the pituitary at a sufficiently high level to prevent up-regulation of GnRH receptor mRNA. The GnRH antagonist, abarelix, is administered as a 100-mg monthly injection to maintain suppression of serum gonadotropins and T (2). The amount of acyline administered to a 70-kg man in this

study as a 300 $\mu\text{g/kg}$ injection was 21 mg, approximately 5 times less than the dose of abarelix. It is therefore possible that a single higher dose of acyline might suppress gonadotropins and T for a month. The only drawback is the volume of acyline that would need to be administered (10.5 ml). A depot formulation of acyline is currently under development by the NICHD.

Adverse side-effects with acyline injection were again minimal, similar to our previous study (9), and included a blush at the injection site and mild pruritus. In this study, however, there was more bruising at the site of injection. There was no pattern to the bruising; it did not occur more commonly for specific individual subjects and was not associated more often with individual nurses who administered the injections. We believe that the bruising probably reflects differences in the manner the injection was administered, rather than being a result of the acyline itself. Three sc nodules at the site of injection were noted in this study: two lasting for 2 d, and one lasting 11 d. Because nodule formation did not occur with every injection in these individuals, these nodules probably represent a tissue reaction to the injection, rather than a reaction to acyline itself.

Other adverse events that occurred during this study in the hypogonadal period were expected as a result of declining T levels. These included hot flashes, decreased libido, fatigue, and irritability, consistent with symptoms of male hypogonadism (19, 20). Because T is known to increase the production of erythropoietin (21, 22), and castration decreases hemoglobin levels (23), our data demonstrating a small, but significant, decrease in hematocrit within the normal range was predictable.

The amount of time for acyline levels in serum to decrease by half ($t_{1/2}$) in this study was 4.9 d, greater in length than the 28.3 h previously found (9). This calculated $t_{1/2}$ for acyline in serum does not fit the classical definition of a true $t_{1/2}$, because it reflects not only the time required for half the total amount of acyline to be cleared from the serum, but also the rate of entry of acyline into serum from the presumed sc depot. Nevertheless, this calculated $t_{1/2}$ allows us to compare data from different studies. The difference found in the $t_{1/2}$ values between the two studies probably reflects the increased number of subjects tested for acyline levels in this study ($n = 7$) vs. the former study ($n = 4$), the similarity in suppression of gonadotropins in the current study (only two of four subjects had suppression of gonadotropins and T for 7 d in the previous study), and the higher dosage of acyline administered in the current study. The long calculated $t_{1/2}$ of acyline might also represent the ability of acyline to bind to serum proteins, as previously discussed (9), or a prolonged time of entry from the sc tissue into the serum compartment secondary to increased volume of injections.

Conclusion

Acyline is a likely candidate for use as a potent, long-lasting GnRH antagonist in the development of an effective male hormonal contraceptive regimen or for the treatment of sex steroid hormone-dependent syndromes, such as advanced severe endometriosis and prostate cancer. It is safe and potentially can be administered as a single dose, twice

a month. Because exogenous long-acting T formulations also inhibit circulating gonadotropins levels and spermatogenesis, a combination of long-acting acyline plus T might make a male hormonal contraceptive regimen a safe, effective, and practical option.

Acknowledgments

We thank Dorothy McGuiness and Arlen Sarkissian for their excellent technical support, and Connie Nosbisch for her efficient coordination of this study.

Received December 11, 2003. Accepted September 1, 2004.

Address all correspondence and requests for reprints to: Dr. Karen L. Herbst, Department of Medicine, Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R. Drew University, Room 3069 Third Floor, 1731 East 120th Street, Los Angeles, California 90059. E-mail: kaherbst@cdrewu.edu.

This work was supported by NIDDK Metabolism Training Grant T32-DK-O7247 (to K.L.H.), the NICHD/NIH through Cooperative Agreement U54-HD-12629 as part of the Specialized Cooperative Centers Program in Reproduction Research and through Cooperative Agreement U54-HD-42454 as part of the Cooperative Contraceptive Research Centers Program. A portion of this work was conducted through the Clinical Research Center facility at the University of Washington and was supported by NIH Grant M01-RR-00037.

References

- Huime JA, Lambalk CB 2001 Gonadotropin-releasing-hormone-receptor antagonists. *Lancet* 358:1793–1803
- Trachtenberg J, Gittleman M, Steidle C, Barzell W, Friedel W, Pessis D, Fotheringham N, Campion M, Garnick MB; Abarelix Study Group 2002 A phase 3, multicenter, open label, randomized study of abarelix versus leuprolide plus daily antiandrogen in men with prostate cancer. *J Urol* 167:1670–1674
- Tarlatzis BC, Bili HN 2003 Gonadotropin-releasing hormone antagonists: impact of IVF practice and potential non-assisted reproductive technology applications. *Curr Opin Obstet Gynecol* 15:259–264
- Felberbaum RE, Kupker W, Diedrich K 2002 Will GnRH antagonists assist in the treatment of benign gynaecological diseases? *Reprod Biomed Online* 5(Suppl 1):68–72
- Kupker W, Felberbaum RE, Krapp M, Schill T, Malik E, Diedrich K 2002 Use of GnRH antagonists in the treatment of endometriosis. *Reprod Biomed Online* 5:12–16
- Roth C 2002 Therapeutic potential of GnRH antagonists in the treatment of precocious puberty. *Expert Opin Invest Drugs* 11:1253–1259
- Wang C, Swerdloff RS 2004 Male hormonal contraception. *Am J Obstet Gynecol* 190:S60–S68
- Anawalt BD, Amory JK 2001 Advances in male hormonal contraception. *Ann Med* 33:587–595
- Herbst KL, Anawalt BD, Amory JK, Bremner WJ 2002 Acyline: the first study in humans of a potent, new gonadotropin-releasing hormone antagonist. *J Clin Endocrinol Metab* 87:3215–3220
- Rivier JE, Jiang G, Porter J, Hoeger CA, Craig AG, Corrigan A, Vale W, Rivier CL 1995 Gonadotropin-releasing hormone antagonists: novel members of the azaline B family. *J Med Chem* 38:2649–2662
- Goldman JM, Cooper RL, Rehnberg GL, Gabel S, McElroy WK, Hein J, Conn PM 1987 Age-related alterations in the stimulated release in vitro of catecholamines and luteinizing hormone-releasing hormone from the male rat hypothalamus. *Neurochem Res* 12:651–657
- Jiang G, Gavini E, Dani BA, Murty SB, Schrier B, Thanoo BC, DeLuca PP 2002 Identification and determination of GnRH antagonist gelling at injection site. *Int J Pharm* 233:19–27
- Kovacs M, Schally AV, Csemes B, Rekasi Z 2001 Luteinizing hormone-releasing hormone (LH-RH) antagonist cetorelix down-regulates the mRNA expression of pituitary receptors for LH-RH by counteracting the stimulatory effect of endogenous LH-RH. *Proc Natl Acad Sci USA* 98:1829–1834
- Tomera K, Gleason D, Gittleman M, Moseley W, Zinner N, Murdoch M, Menon M, Campion M, Garnick MB 2001 The gonadotropin-releasing hormone antagonist abarelix depot versus luteinizing hormone releasing hormone agonists leuprolide or goserelin: initial results of endocrinological and biochemical efficacies in patients with prostate cancer. *J Urol* 165:1585–1589
- Behre HM, Klein B, Steinmeyer E, McGregor GP, Voigt K, Nieschlag E 1992 Effective suppression of luteinizing hormone and testosterone by single doses of the new gonadotropin-releasing hormone antagonist cetorelix (SB-75) in normal men. *J Clin Endocrinol Metab* 75:393–398
- Halmos G, Schally AV, Pinski J, Vellido-Buenfil M, Groot K 1996 Down-

- regulation of pituitary receptors for luteinizing hormone-releasing hormone (LH-RH) in rats by LH-RH antagonist cetrorelix. *Proc Natl Acad Sci USA* 93: 2398–2402
17. Pinski J, Lamharzi N, Halmos G, Groot K, Jungwirth A, Vadillo-Buenfil M, Kakar SS, Schally AV 1996 Chronic administration of the luteinizing hormone-releasing hormone (LHRH) antagonist cetrorelix decreases gonadotrope responsiveness and pituitary LHRH receptor messenger ribonucleic acid levels in rats. *Endocrinology* 137:3430–3436
 18. Kovacs M, Schally AV 2001 Comparison of mechanisms of action of luteinizing hormone-releasing hormone (LHRH) antagonist cetrorelix and LHRH agonist triptorelin on the gene expression of pituitary LHRH receptors in rats. *Proc Natl Acad Sci USA* 98:12197–12202
 19. Seidman SN 2001 Testosterone deficiency and depression in aging men: pathogenic and therapeutic implications. *J Gend Specif Med* 4:44–48
 20. Zitzmann M, Nieschlag E 2000 Hormone substitution in male hypogonadism. *Mol Cell Endocrinol* 161:73–88
 21. Fisher JW, Langston JW 1968 Effects of testosterone, cobalt and hypoxia on erythropoietin production in the isolated perfused dog kidney. *Ann NY Acad Sci* 149:75–87
 22. Naets JP, Wittek M 1968 The mechanism of action of androgens on erythropoiesis. *Ann NY Acad Sci* 149:366–376
 23. Weber JP, Walsh PC, Peters CA, Spivak JL 1991 Effect of reversible androgen deprivation on hemoglobin and serum immunoreactive erythropoietin in men. *Am J Hematol* 36:190–194

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

Testosterone administration to older men improves muscle function: molecular and physiological mechanisms

ARNY A. FERRANDO,¹ MELINDA SHEFFIELD-MOORE,¹ CATHERINE W. YECKEL,¹
CHARLES GILKISON,² JIE JIANG,² ALISON ACHACOSA,³ STEVEN A. LIEBERMAN,²
KEVIN TIPTON,¹ ROBERT R. WOLFE,¹ AND RANDALL J. URBAN²
*Departments of ¹Surgery, ²Internal Medicine, and ³Physical Therapy,
The University of Texas Medical Branch, Galveston, Texas 77550*

Received 25 April 2001; accepted in final form 2 November 2001

Ferrando, Arny A., Melinda Sheffield-Moore, Catherine W. Yeckel, Charles Gilkison, Jie Jiang, Alison Achacosa, Steven A. Lieberman, Kevin Tipton, Robert R. Wolfe, and Randall J. Urban. Testosterone administration to older men improves muscle function: molecular and physiological mechanisms. *Am J Physiol Endocrinol Metab* 282: E601–E607, 2002. First published November 13; 10.1152/ajpendo.00362.2001.—We investigated the effects of 6 mo of near-physiological testosterone administration to older men on skeletal muscle function and muscle protein metabolism. Twelve older men (≥ 60 yr) with serum total testosterone concentrations <17 nmol/l (480 ng/dl) were randomly assigned in double-blind manner to receive either placebo ($n = 5$) or testosterone enanthate (TE; $n = 7$) injections. Weekly intramuscular injections were given for the 1st mo to establish increased blood testosterone concentrations at 1 mo and then changed to biweekly injections until the 6-mo time point. TE doses were adjusted to maintain nadir serum testosterone concentrations between 17 and 28 nmol/l. Lean body mass (LBM), muscle volume, prostate size, and urinary flow were measured at baseline and at 6 mo. Protein expression of androgen receptor (AR) and insulin-like growth factor I, along with muscle strength and muscle protein metabolism, were measured at baseline and at 1 and 6 mo of treatment. Hematological parameters were followed monthly throughout the study. Older men receiving testosterone increased total and leg LBM, muscle volume, and leg and arm muscle strength after 6 mo. LBM accretion resulted from an increase in muscle protein net balance, due to a decrease in muscle protein breakdown. TE treatment increased expression of AR protein at 1 mo, but expression returned to pre-TE treatment levels by 6 mo. IGF-I protein expression increased at 1 mo and remained increased throughout TE administration. We conclude that physiological and near-physiological increases of testosterone in older men will increase muscle protein anabolism and muscle strength.

aging; muscle strength; lean body mass; insulin-like growth factor I

MOST AGING MEN SHOW A REDUCTION in circulating serum testosterone concentrations (16, 22). This reduction in serum testosterone concentration is a core physiological event in what is termed andropause. Andropause

can be clinically characterized by decreased potency and libido, increased fatigability, and decreased muscle strength (13, 24). A significant decrease in serum total testosterone occurs as early as ages 50–59 (16). This decrease in testosterone production is associated with the loss of lean body mass (LBM) and muscle strength. When men are made hypogonadal with a gonadotropin-releasing hormone analog (14), LBM and muscle strength are lost. Once weakened, older individuals are prone to falls that prevent an independent living status and diminish the quality of life. As the population of older Americans grows, the need to develop therapies to counteract the aging-induced loss in skeletal muscle mass and function becomes critically important.

Previously we demonstrated that testosterone administration primes skeletal muscle for growth by increasing net protein synthesis in the fasted state (10, 18). The logical extrapolation of a continued increase in net protein synthesis is an increase in lean body mass and strength. Bhasin et al. (2) demonstrated that supraphysiological doses of testosterone can induce increases in muscle size and strength in younger men without concomitant exercise. This relationship holds true in relatively hypogonadal populations, where the increase of circulating testosterone increases muscle protein synthesis (23), LBM (3, 20), and muscle strength (3, 23). In an earlier study (23), we demonstrated that 1 mo of testosterone administration increased muscle anabolism and strength in six older men. We also demonstrated that the increase in muscle anabolism was associated with an increase in the expression of intramuscular mRNA for insulin-like growth factor I (IGF-I) (23). Because IGF-I has also been demonstrated to be a potent anabolic hormone (11), the relationship between testosterone administration and IGF-I levels was investigated in the present study.

Previous studies of testosterone administration in older men used a standard clinical dosing paradigm (3, 15, 21). Although this dosing is clinically feasible and

Address for reprint requests and other correspondence: A. A. Ferrando, Depts. of Surgery and Metabolism, Shriners Hospitals for Children, 815 Market St., Galveston, TX 77550 (E-mail: aferrando@utmb.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

convenient, it does not account for individual response to hormone administration. We have previously noted that a given dosage of testosterone administration results in widely varied blood concentrations (23). Although group means often reveal significant increases in testosterone, individual variation may mask a consistency in outcomes. For example, Bhasin et al. (3) and Tenover (21) each used a standard clinical replacement dose in elderly men for up to 3 mo. However, Bhasin et al. demonstrated an increase in muscle strength, whereas Tenover did not. Individual response can be resolved in part by using supraphysiological doses (2); however, these doses may be associated with the potential for increased side effects such as altered lipid profiles (12) or hemodynamic profiles (15). In the present study, we endeavored to adjust individual testosterone concentrations to remain within the mid- to high physiological range. We reasoned that remaining within or near physiological testosterone concentrations would diminish potential side effects while allowing the investigation of testosterone's anabolic effects. We hypothesized that increases in testosterone within or near the physiological range would also stimulate muscle anabolism and increase muscle strength in older men much like previous studies where supplementation resulted in supraphysiological concentrations (2, 15). To accomplish this, we carefully adjusted individual nadir hormone concentrations to remain within the physiological range throughout the 6-mo study. This dosing paradigm permits the investigation of the efficacy of long-term testosterone administration at or near physiological concentrations in older men.

METHODS

Subjects. Twelve healthy, older male subjects were randomly assigned in double-blind fashion to receive either testosterone enanthate (TE) or placebo for 6 mo. Seven subjects [68 ± 3 (SE) yr; 91 ± 5 kg] were randomized to receive TE, whereas five subjects (67 ± 3 yr; 99 ± 7 kg) received a placebo consisting of sesame seed oil. The study was approved by the Institutional Review Board at The University of Texas Medical Branch (UTMB). Informed consent was obtained after the study was explained to each individual. Subjects were selected on the basis of the following inclusion criteria: 1) prostate-specific antigen (PSA) ≤ 4.0 $\mu\text{g/l}$ (6), 2) serum total testosterone ≤ 17 nmol/l (480 ng/dl), 3) serum low-density lipoprotein (LDL) ≤ 200 ng/dl (7), 4) completion of a Bruce treadmill exercise test without significant findings of cardiovascular disease, and 5) no medical illnesses causing disability. The serum testosterone cutoff was chosen because it has been shown that 85% of healthy older men (age 60–98 yr) have serum testosterone concentrations < 17 nmol/l but still in the low-normal range of > 10 nmol/l (1). Exclusion criteria included a history of prostate cancer and severe coronary artery disease (due to the possible hypertrophic and atherogenic effects of testosterone), knee replacement (for reasons of strength determination), or use of a blood anticoagulant, e.g., Coumadin (for fear of excessive bleeding during biopsy and catheterization procedures). Because we wanted to determine the outcomes of testosterone without the confounding effects of exercise (2), we excluded subjects engaged in regular training (defined as 30 min of aerobic or resistance

training activity ≥ 2 days/wk). These exclusion/inclusion criteria were similar to those of previously published studies by our group and others (21, 23).

Experimental protocol. The studies were performed at the General Clinical Research Center (GCRC) at UTMB. Subjects were studied at baseline, after 1 mo, and after 6 mo of treatment. Each GCRC admission consisted of ~ 3 days. On day 1, subjects were admitted in the afternoon and underwent Cybex II isokinetic dynamometer testing for muscular endurance. Subjects followed a standardized protocol that included 15 min of pretest stretching. Muscular endurance was defined as the total work performed for 20 repetitions at $240^\circ/\text{s}$. On the morning of day 2, subjects were weighed in hospital gowns, resting (recumbent) blood pressure was taken, and blood was drawn from the fasted subjects for hematological measures. Subjects were then taken for magnetic resonance imaging (MRI) of the lower body. Leg muscle volume was determined by analysis of images collected by MRI (GE Signa 1.5-Tesla whole body imager; General Electric, Milwaukee, WI) as previously described (9). Image data files generated at the MRI facility were analyzed for appendicular total and muscle volumes using NIH Image software (NIH Image public domain analysis package). Muscle volume (cm^3) was computed as the addition of individual slice areas multiplied by the slice thickness (10 mm). After breakfast, subjects were taken to the UTMB Field House for one-repetition maximum (1RM) determinations for bicep curl, tricep extension, leg extension, and leg curl on specific equipment (Cybex) designed for each movement. Subjects were initially familiarized on the equipment after screening and selection. For 1RM testing, subjects first warmed up on a stationary bike set at 30 W for 10 min. The determination of 1RM was accomplished by increasing the load on each machine until successful completion of the movement was no longer possible. The heaviest load lifted was considered the 1RM. At approximately noon, subjects received dual-energy X-ray absorptiometry (DEXA) to determine LBM and fat mass. Body mass components were determined with regional analysis software as previously described (8). Finally, subjects were referred to the Department of Urology at UTMB for prostate ultrasound and urine flow measurements. Prostate volume was measured by transrectal ultrasound, and urinary flow rate measures were made using a Life-Tech uroflowmeter (Life Tech, Houston, TX).

On day 3, subjects received a stable isotope infusion to determine skeletal muscle protein metabolism. Muscle protein net balance and fractional synthesis rate (FSR) of skeletal muscle were determined by infusion of the stable isotope [^3H]ketoisocaproic acid, arteriovenous sampling, and muscle biopsies as previously described (10). Briefly, skeletal muscle FSR was calculated from the determination of the rate of tracer incorporation into the protein and the enrichment of the intracellular pool as the precursor

$$\text{FSR} = [(E_{p2} - E_{p1}) / (E_M \cdot t)] \cdot 60 \cdot 100$$

where E_{p1} and E_{p2} are the enrichments of the protein-bound [^3H]leucine (from transamination of [^3H]ketoisocaproic acid) from the biopsies at 2 and 5 h of isotope infusion; E_M represents the average intracellular [^3H]leucine enrichment over the time of incorporation; and t is the time in minutes. The factors 60 and 100 are required to express FSR in percent per hour. Each biopsy was divided to be used for both Western blot and isotopic enrichment analyses.

After the isotope infusion study on day 3, subjects were given injections and discharged. Subjects returned every week for fasted blood draw and injections for the first 4 wk and then every 2 wk for the remainder of the study. Serum

total testosterone concentrations were measured on each occasion and adjusted to between 17 and 28 nmol/l (500 and 800 ng/dl; based on the concentration for the visit before each injection) to approximate concentrations found in young men. The aforementioned measurements were made at baseline and at 1 and 6 mo. However, at 1 mo, the MRI, DEXA, and urology measures were omitted. We designed the TE dosing paradigm for weekly injections for the 1st mo so that we could adjust TE doses and establish increased testosterone concentrations by the first measurements that were done at 1 mo. This paradigm was reproduced from our initial study (23). A biweekly injection paradigm would not have allowed TE dose adjustment before the assessments at 1 mo.

Clinical measures. Measurement of clinical parameters (see Table 2) such as testosterone (DPC, Los Angeles, CA), estradiol (DPC), blood lipids (Vitros 250 Chemistry System, Johnson & Johnson, Arlington, TX), PSA, liver function tests (Vitros 250), and hematocrit (Coutter Onyx, Beckman Coulter, Brea, CA) were done on a monthly basis by a UTMB clinical laboratory. Subjects were also monitored monthly for breast tenderness and the presence of gynecomastia by history and physical examination. Serum testosterone concentrations were determined by the clinical laboratory, so that adjustments in TE doses could be made on the basis of the previous serum testosterone concentration.

Western blot analysis. Protein was isolated from muscle biopsy samples by slicing frozen muscle in very small pieces with a clean razor blade and thawing the tissue in lysis buffer (150 mM NaCl, 10 mM Tris, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 5 mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 10 μ g/ml aprotinin, 50 μ g/ml leupeptin, 1 μ g/ml pepstatin A) at a concentration of 3 ml of ice cold lysis buffer per gram of tissue. The tissue was homogenized with a Dounce homogenizer (4°C) and centrifuged at 15,000 *g* for 20 min, and the supernatant was removed and centrifuged again to result in total cell lysate. The androgen receptor (AR) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 80 mg of cell lysate run on standard SDS-PAGE gel with a working solution concentration range of 1:15–20. The IGF-I antibody (Santa Cruz Biotechnology) was incubated with 40 mg of cell lysate run on standard SDS-PAGE gel with a working solution concentration of 1:100. The actin “housekeeping” antibody (Sigma) was used with a working solution concentration range of 1:100–200. This anti-actin antibody is a broad-based antibody that recognizes an epitope located on the NH₂-terminal region of actin and demonstrates a broad reactivity among multiple actin isoforms in various species. The housekeeping antibody was used to correct the results for protein loading of the gel. Western analysis allows the direct measurement of protein expression in the muscle biopsy samples.

Statistical analysis. Comparison of 1- and 6-mo measures to baseline values was accomplished by 2-way repeated-measures ANOVA with Dunnett's multiple comparison test. Comparison of clinical outcome values over the 6-mo study period was accomplished by ANOVA with Dunnett's multiple comparison test. Where 1-mo measures were omitted, a paired *t*-test was used to statistically compare 6-mo and baseline values. Statistical significance was $P \leq 0.05$. Data are presented as means \pm SE.

RESULTS

Clinical outcomes. Figure 1 shows the mean testosterone profiles of each group at 2-wk intervals over the 6-mo study period. Table 1 shows the individual tes-

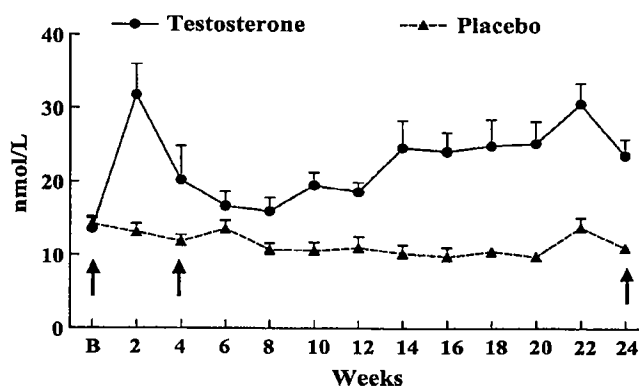


Fig. 1. Serum testosterone profiles throughout treatment. Values are means \pm SE. Testosterone treatment group values were significantly higher at all time points after baseline. Arrows indicate study time points.

tosterone concentrations for each of the seven subjects who received TE and the dose adjustment made for each individual. None were clinically hypogonadal at the beginning of this study. TE injections were adjusted by an independent clinician to maintain levels within the normal range (17–28 nmol/l). As can be seen in Table 1, the serum testosterone concentrations and the doses of TE administered were variable from individual to individual. Following such a paradigm, especially with the use of intramuscular injections, the older men were exposed to serum testosterone concentrations at various times during the 6-mo study that were above the physiological range. Therefore, this study assesses a mix between physiological and near-physiological administration. However, serum testosterone concentrations were greater in the treatment group at all time points after baseline ($P < 0.05$). Serum testosterone did not change in the placebo group. Table 2 delineates subject characteristics and laboratory values over the 6-mo study period. Treatment subjects remained normotensive, and liver function tests, blood lipid profiles, and PSA were unchanged. Estradiol increased upon treatment and, for the most part, remained elevated throughout the 6-mo period without causing breast tenderness or gynecomastia by report or examination. Hematocrit was elevated after 4 mo of TE and remained elevated until the end of the study.

Prostate volume was not significantly increased with TE administration. Prostate volume in the treatment group was 44 ± 15 ml at baseline, whereas the placebo group was 41 ± 8 ml. Six-month values were 47 ± 13 and 35 ± 7 ml, respectively, for the treatment and placebo groups. Urinary flow rate also did not change over time or as a result of treatment. Baseline flow rate was 8.3 ± 1.5 and 8.9 ± 1.3 ml/s, whereas 6-mo values were 7.5 ± 1.4 and 8.7 ± 1.6 ml/s for the treatment and placebo groups, respectively.

Western blot analysis. TE administration significantly increased skeletal muscle AR protein expression at 1 mo ($P < 0.05$), but AR returned to baseline levels at 6 mo. Figure 2 shows a representative autoradio-

Table 1. Serum testosterone concentrations and TE dose adjustments for the 7 older men receiving testosterone

| S | Baseline | 1 wk | 2 wk | 3 wk | 4 wk | 6 wk | 8 wk | 10 wk | 12 wk | 14 wk | 16 wk | 18 wk | 20 wk | 22 wk | 24 wk |
|---|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|-------|
| 1 | 10.7 (100) | 30.9 (100) | 30.9 (100) | 26.3 (100) | 43.3 (150) | 17.6 (125) | 15.8 (200) | 7.4 (200) | 23.7 (200) | 23.9 (200) | 20.6 (200) | 21.0 (250) | 16.4 (250) | 36.9 (300) | 31.8 |
| 2 | 13.9 (100) | 21.8 (100) | 22.1 (100) | 24.1 (50) | 27.0 (150) | 13.3 (150) | 16.5 (150) | 15.4 (200) | 17.6 (200) | 13.9 (250) | 23.2 (250) | 13.9 (300) | 12.2 (350) | 17.5 (400) | 29.3 |
| 3 | 15.9 (100) | 32.3 (100) | 36.8 (100) | 33.6 (50) | 22.4 (150) | 10.7 (150) | 12.6 (200) | 14.7 (200) | 17.6 (250) | 18.4 (250) | 20.1 (300) | 21.9 (300) | 21.8 (350) | 17.1 (350) | 21.2 |
| 4 | 13.3 (100) | 20.5 (100) | 27.8 (150) | 32.8 (150) | 50.3 (150) | 45.5 (150) | 13.9 (100) | 18.2 (200) | 20.6 (200) | 20.7 (200) | 22.1 (250) | 28.6 (250) | 40.2 (250) | 26.6 (200) | 26.4 |
| 5 | 11.4 (100) | 24.4 (100) | 22.2 (100) | 19.6 (100) | 24.0 (200) | 9.4 (200) | 12.0 (250) | 14.8 (300) | 21.0 (350) | 18.1 (350) | 22.0 (400) | 30.3 (400) | 25.6 (400) | 26.8 (400) | 37.3 |
| 6 | 9.6 (100) | 13.8 (100) | 18.7 (150) | 26.6 (150) | 22.3 (200) | 23.8 (200) | 19.4 (200) | 18.9 (250) | 11.4 (250) | 14.9 (300) | 17.9 (300) | 21.5 (400) | 24.5 (400) | 18.2 (400) | 25.4 |
| 7 | 13.2 (100) | 16.8 (100) | 22.1 (150) | 29.8 (150) | 33.0 (200) | 20.2 (200) | 26.6 (250) | 22.6 (250) | 25.0 (300) | 20.6 (300) | 46.3 (300) | 32.7 (300) | 33.6 (300) | 32.9 (300) | 42.7 |

Nos. are testosterone concentrations in nmol/l; nos. in parentheses are doses of testosterone enanthate (TE) administered (mg) at that visit. S, subject. Testosterone dose adjustments were made on the basis of the preceding testosterone concentrations; i.e., an adjustment at 8 wk was based on the 6-wk testosterone concentration.

gram of a Western blot for skeletal muscle AR from a subject receiving testosterone and a graph of the densitometry data from the treatment group. There was no correlation between the serum testosterone concentration at 1 mo and the change of AR expression from baseline to 1 mo for individuals. IGF-I protein expression in skeletal muscle increased at 1 mo and remained elevated at 6 mo ($P < 0.05$; Fig. 3). AR and IGF-I protein expression did not change in the placebo group (data not shown).

Physiological outcomes. The net balance of muscle protein was less negative in the fasted state in the treatment group throughout TE administration (Fig. 4; $P < 0.05$), but still less than zero. In other words,

treatment subjects were less catabolic when fasting than those in the placebo group. The more favorable net balance was due to a decrease in fasting protein breakdown, as fractional synthetic rate of muscle protein remained constant throughout (0.071 ± 0.02 to 0.084 ± 0.013 to $0.062 \pm 0.016\%/h$ at baseline and 1 and 6 mo, respectively).

The resultant improvement in net protein balance led to an increase in LBM. Table 3 outlines the changes in LBM and muscle strength over the 6-mo study period. The treatment group demonstrated increases in total and leg LBM, whereas the percentage of total body fat diminished. Leg muscle volume by MRI was also increased significantly after 6 mo of TE adminis-

Table 2. Subject characteristics and laboratory values during 6 mo of testosterone or placebo treatment

| | Baseline | 1 mo | 2 mo | 3 mo | 4 mo | 5 mo | 6 mo |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Weight, kg | 91 \pm 5 | 92 \pm 5 | 92 \pm 5 | 92 \pm 5 | 92 \pm 5 | 92 \pm 5 | 91 \pm 6 |
| Systolic BP, mmHg | 99 \pm 7 | 98 \pm 6 | 98 \pm 7 | 98 \pm 7 | 96 \pm 6 | 96 \pm 6 | 96 \pm 6 |
| Diastolic BP, mmHg | 141 \pm 7 | 144 \pm 4 | 154 \pm 5 | 150 \pm 4 | 156 \pm 5 | 152 \pm 6 | 149 \pm 3 |
| ALT U/l (9–51) | 140 \pm 8 | 148 \pm 5 | 155 \pm 3 | 150 \pm 7 | 154 \pm 4 | 147 \pm 8 | 148 \pm 5 |
| AST U/l (13–40) | 78 \pm 3 | 79 \pm 3 | 82 \pm 4 | 85 \pm 4 | 87 \pm 3 | 84 \pm 3 | 85 \pm 3 |
| Chol. (mmol/l) (3.1–5.2) | 82 \pm 6 | 81 \pm 7 | 79 \pm 5 | 83 \pm 5 | 83 \pm 6 | 78 \pm 5 | 79 \pm 4 |
| HDL (mmol/l) (0.78–1.8) | 32 \pm 3 | 30 \pm 2 | 27 \pm 2 | 27 \pm 3 | 29 \pm 3 | 32 \pm 3 | 28 \pm 3 |
| LDL (mmol/l) (2.1–5.69) | 36 \pm 10 | 37 \pm 9 | 35 \pm 6 | 34 \pm 6 | 33 \pm 6 | 29 \pm 2 | 30 \pm 4 |
| PSA, μ g/l (<4) | 23 \pm 2 | 23 \pm 1 | 24 \pm 3 | 22 \pm 2 | 24 \pm 2 | 25 \pm 2 | 24 \pm 2 |
| E ₂ , pmol/l (48–173) | 24 \pm 7 | 27 \pm 7 | 25 \pm 6 | 25 \pm 5 | 24 \pm 5 | 19 \pm 5 | 22 \pm 5 |
| Hct, % (37–50) | 4.81 \pm 0.62 | 4.81 \pm 0.28 | 4.71 \pm 0.21 | 4.65 \pm 0.28 | 4.73 \pm 0.16 | 4.84 \pm 0.16 | 4.50 \pm 0.23 |
| | 5.15 \pm 0.34 | 5.17 \pm 0.44 | 5.30 \pm 0.31 | 5.3 \pm 0.26 | 4.84 \pm 0.28 | 5.02 \pm 0.26 | 5.12 \pm 0.26 |
| | 1.14 \pm 0.13 | 1.01 \pm 0.10 | 1.11 \pm 0.13 | 1.06 \pm 0.10 | 1.06 \pm 0.13 | 1.09 \pm 0.13 | 0.96 \pm 0.13 |
| | 0.88 \pm 0.08 | 0.96 \pm 0.08 | 0.91 \pm 0.05 | 0.88 \pm 0.05 | 0.85 \pm 0.08 | 0.93 \pm 0.08 | 0.88 \pm 0.08 |
| | 2.77 \pm 0.34 | 2.79 \pm 0.28 | 2.79 \pm 0.18 | 2.71 \pm 0.26 | 2.77 \pm 0.6 | 2.92 \pm 0.18 | 2.69 \pm 0.23 |
| | 2.97 \pm 0.54 | 3.23 \pm 0.47 | 3.36 \pm 0.39 | 2.92 \pm 0.57 | 2.82 \pm 0.47 | 3.02 \pm 0.34 | 3.05 \pm 0.40 |
| | 1.4 \pm 0.4 | 1.9 \pm 0.4 | 2.1 \pm 0.7 | 2.0 \pm 0.7 | 2.1 \pm 0.4 | 2.0 \pm 0.5 | 2.3 \pm 0.9 |
| | 1.2 \pm 0.4 | 1.4 \pm 0.4 | 1.2 \pm 0.4 | 1.0 \pm 0.4 | 1.1 \pm 0.4 | 1.2 \pm 0.3 | 1.3 \pm 0.4 |
| | 103 \pm 7 | 272 \pm 33* | 114 \pm 1 | 154 \pm 26* | 187 \pm 26* | 169 \pm 18* | 242 \pm 40* |
| | 110 \pm 15 | 14 \pm 7 | 92 \pm 15 | 103 \pm 18 | 92 \pm 11 | 103 \pm 11 | 117 \pm 15 |
| | 40 \pm 0.8 | 40 \pm 0.7 | 43 \pm 0.8 | 44 \pm 0.9 | 45 \pm 0.1* | 46 \pm 0.8* | 44 \pm 1* |
| | 40 \pm 0.7 | 41 \pm 0.3 | 41 \pm 0.6 | 43 \pm 0.7 | 42 \pm 1 | 41 \pm 0.4 | 40 \pm 0.8 |

Values are means \pm SE. For each test parameter, the first line represents values for the testosterone group ($n = 7$), and the second line represents values for the placebo group ($n = 5$). BP, blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Chol., total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PSA, prostate-specific antigen; E₂, estradiol; Hct, hematocrit. Normal ranges are given in parentheses by the tests. *Statistical significance from the placebo group at each time point as determined by ANOVA with Dunnett's multiple comparison test.

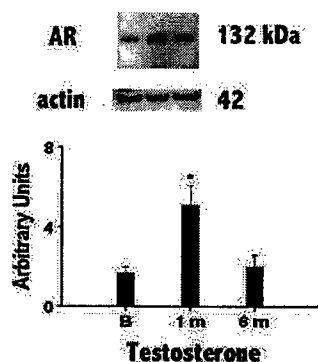


Fig. 2. Androgen receptor (AR) protein expression in skeletal muscle during 6 mo of testosterone administration in older men. *Top*: representative Western blot from one of the 7 subjects assessed for protein expression of AR by use of standard Western analysis. Actin was used as an internal control for protein loading. *Bottom*: means \pm SE from the 7 subjects that received testosterone. Five subjects who received placebo demonstrated no change throughout the study in AR expression (data not shown). Data are expressed as arbitrary units calculated as the ratio of the band densities of AR over the band densities of actin. *Statistical significance was determined by ANOVA, $P \leq 0.05$.

tration. All 1RM strength scores increased in the treatment group after 6 mo of TE. Muscular endurance, as tested by an isokinetic dynamometer, did not increase at 1 or 6 mo.

DISCUSSION

This study demonstrates that testosterone increases within or near the physiological range can produce increases in muscle anabolism, LBM, and muscle strength similar to supraphysiological administration. We monitored serum testosterone concentrations and adjusted the dose of TE to maintain testosterone concentrations in older men in ranges comparable with those of younger men. During the 6 mo of TE administration, some subjects experienced testosterone concentrations that exceeded the physiological; however,

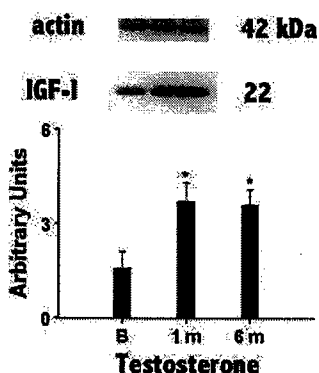


Fig. 3. Insulin-like growth factor I (IGF-I) protein expression in skeletal muscle during 6 mo of testosterone administration in older men. *Top*: representative Western blot from one of 7 subjects assessed for expression of IGF-I by use of standard Western analysis. *Bottom*: mean data from the 7 subjects receiving testosterone administration. Five subjects who received placebo demonstrated no change throughout the study in IGF-I expression (data not shown). Data were derived as described in Fig. 2.

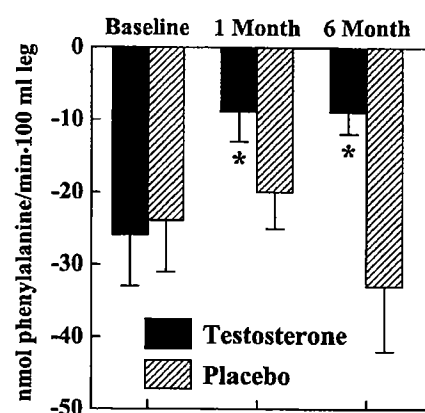


Fig. 4. Fasting net phenylalanine balance across the leg. Phenylalanine net balance describes the net balance between muscle protein synthesis and breakdown. *Significantly less negative than the placebo group and baseline testosterone, by ANOVA, $P < 0.05$.

testosterone concentrations were consistently maintained above baseline values. The older men in this study demonstrated an increase in LBM that was comparable to that achieved with a standard replacement regimen that resulted in higher testosterone concentrations (5). We also found that, similar to younger men (2), testosterone will increase muscle anabolism and strength in older men. The strength increases of the older men in this study were greater than those demonstrated with standard replacement paradigms (15, 21) or with testosterone patch administration over

Table 3. Absolute changes in body mass and muscle strength with 6 mo of testosterone treatment

| Variable, units | Treatment | | P Value |
|--|--------------------------|-------------------------------|----------|
| | Testosterone | Placebo | |
| ¹ Total LBM, kg | $\uparrow 4.2 \pm 0.6$ | $\downarrow 2.0 \pm 1.0$ | <0.001 |
| ¹ Leg LBM, kg | $\uparrow 1.6 \pm 0.4$ | $\downarrow 1.3 \pm 0.6$ | 0.003 |
| ¹ Arm LBM, kg | $\uparrow 1.6 \pm 0.6$ | $\downarrow 1.4 \pm 1.4$ | 0.056 |
| ¹ Body fat, % | $\downarrow 3.6 \pm 0.7$ | $\uparrow 0.3 \pm 1.7$ | 0.039 |
| ² Leg muscle volume, ml | $\uparrow 488 \pm 76$ | $\downarrow 96 \pm 155$ | 0.04 |
| ³ Bicep curl, kg | | | |
| 1 mo | $\uparrow 3.6 \pm 2.3$ | $\downarrow 0.5 \pm 2.0$ | 0.16 |
| 6 mo | $\uparrow 9.1 \pm 1.5$ | $\downarrow 0.9 \pm 1.5$ | 0.002 |
| ³ Tricep extension, kg | | | |
| 1 mo | $\uparrow 4.9 \pm 1.7$ | $\uparrow 2.3 \pm 1.6$ | 0.33 |
| 6 mo | $\uparrow 10.4 \pm 2.1$ | $\downarrow 0.9 \pm 1.2$ | <0.001 |
| ³ Leg curl, kg | | | |
| 1 mo | $\uparrow 5.5 \pm 2.4$ | $\uparrow 1.8 \pm 1.3$ | 0.11 |
| 6 mo | $\uparrow 7.5 \pm 2.1$ | $\leftrightarrow 0.0 \pm 4.0$ | 0.051 |
| ³ Leg extension, kg | | | |
| 1 mo | $\uparrow 6.5 \pm 3.1$ | $\uparrow 4.5 \pm 1.4$ | 0.73 |
| 6 mo | $\uparrow 15.3 \pm 5.2$ | $\leftrightarrow 0.0 \pm 3.1$ | 0.015 |
| Isokinetic endurance knee extension, dominant leg, J | | | |
| 1 mo | $\uparrow 2.3 \pm 3.1$ | $\uparrow 3.4 \pm 6.8$ | .313 |
| 6 mo | $\uparrow 17.6 \pm 9.8$ | $\uparrow 6.0 \pm 7.5$ | .344 |

Values are means \pm SE. LBM, lean body mass. ¹Dual-energy X-ray absorptiometry; ²magnetic resonance imaging; ³one-repetition maximum on Cybex equipment with both limbs. P value denotes differences between treatments; \uparrow or \downarrow indicates direction of numerical change.

36 mo (20). Our data suggest that a standard paradigm of testosterone administration that does not include individual dose adjustment may not always achieve desired outcomes if the subjects have not received adequate testosterone to stimulate metabolic changes in muscle. Because we studied only a small number of subjects, we cannot draw any conclusions regarding the risk-to-benefit ratio of testosterone administration in older men. However, we found no significant side effects in our small group other than an increase in hematocrit. Our data indicate that testosterone can improve muscle strength in older men when careful dosing ensures sustained blood testosterone increases. Our first study demonstrated that short-term administration with standard replacement dosages resulted in LBM and strength increases (23). The present study indicates that these LBM and strength increases can be maintained over 6 mo with careful dose adjustments that ensure primarily physiological testosterone levels. This study also demonstrates that the muscle's response to testosterone changes over the 6-mo period of administration, indicating that alternative paradigms of testosterone administration (i.e., cyclic administration) can be of physiological benefit.

Testosterone administration resulted in some noteworthy effects on AR and IGF-I expression in skeletal muscle. AR protein expression was increased after 1 mo of TE but had returned to pretreatment levels by 6 mo. Physiologically, it is logical that androgen would enhance its own receptor expression as it stimulates muscle metabolism. We previously noted an upregulation of AR expression with oxandrolone administration (18) in young males, which also occurred concomitantly with an increase in muscle protein synthesis. The return of AR expression to pretreatment values after 6 mo of continuous androgen administration indicates a steady-state adaptation to the treatment paradigm. There is also the possibility that the AR response is nothing more than a response to the dosing paradigm. At 1 mo, older subjects were receiving TE weekly rather than every 2 wk, and their mean serum testosterone concentrations were more in the supraphysiological range than they were at 6 mo. However, this relationship is weakened by the fact that individual testosterone concentrations at 1 mo did not correlate with the change in AR expression from baseline to 1 mo. This pattern of AR expression raises the possibility that cycling of testosterone administration could produce effects on skeletal muscle analogous to continuous administration. Such a paradigm would be beneficial by administering significantly less testosterone for similar anabolic outcomes, thus minimizing the possibility of side effects.

IGF-I accompanies increases in muscle mass and strength (17). In frail elderly, progressive resistance training that increases muscle mass and strength also increases intramuscular IGF-I concentrations (19). Clinically, we previously demonstrated that older men given testosterone for 1 mo increased IGF-I transcripts in muscle while decreasing the inhibitory IGF-binding protein (23). The present study agrees with our previ-

ous work in that IGF-I protein expression increased at 1 mo and further demonstrates that this increase was maintained throughout the 6 mo of testosterone administration. This confirms that the increase in IGF-I mRNA noted in our earlier study (23) translates into an actual increase of IGF-I protein. A corollary to these studies found that young men who were made hypogonadal for 10 wk by Lupron showed a decrease in muscle strength and a decrease in intramuscular IGF-I mRNA concentration (14). Taken together, these data indicate a mechanistic importance of IGF-I on muscle anabolism.

Although the intracellular mechanism stimulating muscle protein anabolism requires further clarification, it is clear that testosterone improves net protein balance of skeletal muscle. This effect is pronounced in the fasted state as net protein balance becomes less negative. We have previously demonstrated (10, 18) that one of the primary effects of testosterone (during fasting) is the efficient reutilization of intracellular amino acids (derived from protein breakdown) for protein synthesis. However, the present study demonstrates that, even if breakdown is decreased, ample amino acid precursors are present to support the initial rate of protein synthesis. Thus testosterone administration may ameliorate the loss of skeletal muscle nitrogen during fasting in this older population by preventing the loss of intracellular amino acids. Not only is the appearance of amino acids from protein breakdown reduced, but those that are derived from protein breakdown are efficiently utilized to maintain protein synthesis, as we have previously demonstrated (10, 18). This retention of nitrogen during fasting, when combined with the anabolic stimulus of a meal alone (4, 25), may lead to muscle (LBM) accretion over time and explain the anabolic effects of chronic testosterone administration.

In summary, the present study demonstrates that careful and near-physiological testosterone administration in older men will increase LBM and muscle strength similarly to younger men. However, further consideration should be given to the specific androgen and length and type of administration regimen to be used in older men and to large-scale studies initiated to determine the risk-to-benefit ratio of testosterone administration in older men.

This study was supported by National Institutes of Health Grants AG/AR-11000 (R. J. Urban), M01-RR-00073 (General Clinical Research Center, University of Texas Medical Branch), GM-57295 (A. A. Ferrando), and Shriners Hospitals for Children Grant 8940 (R. R. Wolfe).

REFERENCES

1. Abbasi AA, Drinka PJ, Mattson DE, and Rudman D. Low circulating levels of insulin-like growth factors and testosterone in chronically institutionalized elderly men. *J Am Geriatr Soc* 41: 975-982, 1993.
2. Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Tricker R, Shirazi A, and Casaburi R. The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med* 335: 1-7, 1996.

3. Bhasin S, Storer TW, Berman N, Yarasheski KE, Clevenger B, Phillips J, Lee WP, Bunnell TJ, and Casaburi R. Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. *J Clin Endocrinol Metab* 82: 407–413, 1997.
4. Biolo G, Tessari P, Inchiostro S, Bruttomesso D, Fongher C, Sabadin L, Fratton MG, Valerio A, and Tiengo A. Leucine and phenylalanine kinetics during mixed-meal ingestion: a multiple tracer approach. *Am J Physiol Endocrinol Metab* 262: E455–E463, 1992.
5. Brodsky IG, Balagopal P, and Nair KS. Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men—a clinical research center study. *J Clin Endocrinol Metab* 81: 3469–3475, 1996.
6. Catalona WJ, Smith DS, Ratliff TL, Dodds KM, Coplen DE, Yuan JJ, Petros JA, and Andriole GL. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer [published erratum appears in *N Engl J Med* 1991 Oct 31, 325: 1324]. *N Engl J Med* 324: 1156–1161, 1991.
7. Denke MA and Grundy SM. Hypercholesterolemia in elderly persons: resolving the treatment dilemma. *Ann Intern Med* 112: 780–792, 1990.
8. Ferrando AA, Lane HW, Stuart CA, Davis-Street J, and Wolfe RR. Prolonged bed rest decreases skeletal muscle and whole body protein synthesis. *Am J Physiol Endocrinol Metab* 270: E627–E633, 1996.
9. Ferrando AA, Stuart CA, Brunder DG, and Hillman GR. Magnetic resonance imaging quantitation of changes in muscle volume during 7 days of strict bed rest. *Aviat Space Environ Med* 66: 976–981, 1995.
10. Ferrando AA, Tipton KD, Doyle D, Phillips SM, Cortiella J, and Wolfe RR. Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. *Am J Physiol Endocrinol Metab* 275: E864–E871, 1998.
11. Fryburg DA, Jahn LA, Hill SA, Oliveras DM, and Barrett EJ. Insulin and insulin-like growth factor-I enhance human skeletal muscle protein anabolism during hyperaminoacidemia by different mechanisms. *J Clin Invest* 96: 1722–1729, 1995.
12. Haupt HA and Rovere GD. Anabolic steroids: a review of the literature. *Am J Sports Med* 12: 469–484, 1984.
13. Mastrogiacomo I, Feghali G, Foresta C, and Ruzza G. Andropause: incidence and pathogenesis. *Arch Androl* 9: 293–296, 1982.
14. Mauras N, Hayes V, Welch S, Rini A, Helgeson K, Dokler M, Veldhuis JD, and Urban RJ. Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. *J Clin Endocrinol Metab* 83: 1886–1892, 1998.
15. Morley JE, Perry HM III, Kaiser FE, Kraenzle D, Jensen J, Houston K, Mattammal M, and Perry HM Jr. Effects of testosterone replacement therapy in old hypogonadal males: a preliminary study. *J Am Geriatr Soc* 41: 149–152, 1993.
16. Moroz EV and Verkhatsky NS. Hypophyseal-gonadal system during male aging. *Arch Gerontol Geriatr* 4: 13–19, 1985.
17. Sheffield-Moore M. Androgens and the control of skeletal muscle protein synthesis. *Ann Med* 32: 181–186, 2000.
18. Sheffield-Moore M, Urban RJ, Wolf SE, Jiang J, Catlin DH, Herndon DN, Wolfe RR, and Ferrando AA. Short-term oxandrolone administration stimulates net muscle protein synthesis in young men. *J Clin Endocrinol Metab* 84: 2705–2711, 1999.
19. Singh MA, Ding W, Manfredi TJ, Solares GS, O'Neill EF, Clements KM, Ryan ND, Kehayias JJ, Fielding RA, and Evans WJ. Insulin-like growth factor I in skeletal muscle after weight-lifting exercise in frail elders. *Am J Physiol Endocrinol Metab* 277: E135–E143, 1999.
20. Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Lenrow DA, Holmes JH, Dlewati A, Santanna J, Rosen CJ, and Strom BL. Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. *J Clin Endocrinol Metab* 84: 2647–2653, 1999.
21. Tenover JS. Effects of testosterone supplementation in the aging male. *J Clin Endocrinol Metab* 75: 1092–1098, 1992.
22. Tenover JS, Matsumoto AM, Plymate SR, and Bremner WJ. The effects of aging in normal men on bioavailable testosterone and luteinizing hormone secretion: response to clomiphene citrate. *J Clin Endocrinol Metab* 65: 1118–1126, 1987.
23. Urban RJ, Bodenbun YH, Gilkison C, Foxworth J, Coggan AR, Wolfe RR, and Ferrando A. Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. *Am J Physiol Endocrinol Metab* 269: E820–E826, 1995.
24. Urban RJ and Veldhuis VJ. Hypothalamo-pituitary concomitants of aging. In: *The Endocrinology of Aging*, edited by Sowers JR and Felicetta JV. New York: Raven, 1988, p. 44–74.
25. Volpi E, Mittendorfer B, Wolf SE, and Wolfe RR. Oral amino acids stimulate muscle protein anabolism in the elderly despite higher first-pass splanchnic extraction. *Am J Physiol Endocrinol Metab* 277: E513–E520, 1999.

Oral Testosterone in Oil Plus Dutasteride in Men: A Pharmacokinetic Study

John K. Amory and William J. Bremner

Center for Research in Reproduction and Contraception, Divisions of General Internal Medicine and Endocrinology, Metabolism, and Nutrition, University of Washington Medical School, Seattle, Washington 98195

Testosterone (T) is not administered orally, because it has been reported to be rapidly metabolized by the liver. We hypothesized that sufficient doses of T or T enanthate (TE), administered orally in oil, would result in clinically useful elevations in serum T. We also hypothesized that coadministration of dutasteride (D) with T or TE would minimize increases in serum DHT seen previously with oral administration. Therefore, we conducted a pharmacokinetic study of oral T and TE in oil, with and without concomitant D, in normal men whose T production had been temporarily suppressed by the GnRH antagonist acyline. Thirteen healthy men (mean age, 24 ± 6 yr) were enrolled and assigned to oral T ($n = 7$) and oral TE ($n = 6$) groups and were administered 200, 400, or 800 mg of either T or TE in sesame oil in the morning on 3 successive days 24 h after receiving acyline. Blood samples for measurement of serum T and dihydrotestos-

terone were obtained before T or TE administration and 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h after administration. Subjects were then administered D for 4 d before repeating the sequence of T or TE doses with D. Serum T was significantly increased in a dose-dependent fashion with the administration of oral T or TE in oil. Coadministration of D with oral T or TE significantly increased the 24-hr average serum T levels compared with administration of T or TE alone [average serum T after 400 mg dose, 8.7 ± 3.0 nmol/l (T) and 8.3 ± 5.7 nmol/l (TE) vs. 16.1 ± 5.8 nmol/l (T + D) and 15.0 ± 8.8 nmol/l (TE + D); $P < 0.05$ for T vs. T and D]. The administration of oral T or TE in oil combined with D results in unexpected and potentially therapeutic increases in serum T. Additional studies of this combination as a novel form of oral androgen therapy are warranted. (*J Clin Endocrinol Metab* 90: 2610–2617, 2005)

TESTOSTERONE (T) IS crucial for male health. The normal male testes produce 4–8 mg T daily (1, 2). Depending on age, 2.5–10% of men have T levels below the normal range (3). T has effects on a variety of tissues, including brain, liver, muscle, bone and bone marrow, blood vessels, skin, prostate, and penis. Men with T deficiency have symptoms of depression, reduced libido, and low energy and suffer from anemia, osteoporosis, and debilitating muscle weakness. These men require T replacement therapy to improve well-being, maintain bone and muscle mass, and retain healthy sexual function (4–8), yet there is no acceptable form of oral T for therapy in the United States.

Most T regimens in the United States depend on parenteral injections, skin patches, gels, or buccal tablets (9–11), because currently available oral forms of T are alkylated and cause liver toxicity when used long term (11–16). Injections are administered im every 1–3 wk and can be painful (17). Some T patches can cause moderate to severe skin reactions due to the vehicle that facilitates T absorption across the skin (18). The T gels are effective and generally well accepted by patients, but are expensive, and care must be taken to avoid inadvertent exposure to women and children (19).

Oral administration of unmodified T at doses up to 100 mg have little effect on serum T levels in T-deficient men (20, 21); however, 200-mg doses of oral T have been shown to elevate

serum T levels to the low normal range for up to 8 h (22, 23). At the time, these serum T levels were thought to be insufficient for clinical use, and research into using unmodified oral T was largely abandoned.

Testosterone undecanoate (TU) is a T ester currently given orally in oil and used clinically in Europe and Canada for the treatment of T deficiency. When administered orally, TU therapy results in therapeutic increases in serum T; however, it also results in elevations in serum dihydrotestosterone (DHT) well above the normal range (24–27). Because DHT is required for cell growth within the prostate, concern has been raised about the potential for long-term harm associated with oral TU therapy from the elevated levels of serum DHT; however, no increased risk of prostate disease has been reported to date.

Because the androgen TU is absorbed well in oil, we believed that other androgens such as T enanthate (TE) and potentially T itself might be well absorbed if also administered orally in oil. Moreover, because the recently available 5α -reductase inhibitor, dutasteride (D), lowers serum DHT levels more than 90% by inhibiting both isozymes of 5α -reductase (28), we hypothesized that oral administration of the combination of higher doses of unmodified T or the T ester, TE, in oil when combined with D would be safe and result in therapeutic serum T levels. In addition, we hypothesized that the concomitant administration of the 5α -reductase inhibitor D with T or TE would further increase serum T levels while minimizing the elevations in serum DHT seen after oral administration of oral androgens such as TU. If effective, we believed that this novel means of T therapy would allow for selective androgen therapy in men with T

First Published Online February 15, 2005

Abbreviations: D, Dutasteride; DHT, dihydrotestosterone; E2, estradiol; T, testosterone; $t_{1/2}$, half-life; TE, testosterone enanthate; TU, testosterone undecanoate.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

deficiency. Therefore, we conducted a pilot study of the oral administration of single doses of T and TE with and without concomitant administration of D to determine the pharmacokinetics and safety of single high doses oral T in oil in healthy men rendered temporarily hypogonadal with the GnRH antagonist acyline.

Materials and Methods

Subjects

Fourteen healthy, normal male volunteers between 18 and 45 yr of age were recruited through local news media (newspaper and radio) and college campus bulletin boards and enrolled in the study. The inclusion criteria were no prior medical illnesses, normal physical examination, and routine hematology, blood chemistry, and liver function. Exclusion criteria included regular use of any medication; abnormal serum T, DHT, or estradiol (E2); or previous or current ethanol, illicit drug, or anabolic steroid abuse. A total of 16 men were evaluated for eligibility. Of these, 14 men were potentially eligible and agreed to participate in the study. The two men who did not enroll in the study were excluded for elevated bilirubin (one subject) and use of finasteride (for the treatment of male-pattern baldness). One enrolled subject failed to appear for his acyline injection and was therefore not studied further; thus, 13 men completed the study period. The institutional review board of University of Washington approved all study procedures, and subjects gave written informed consent before screening.

Study design

Participants were randomly assigned to one of two groups: 1) oral T in sesame oil, or 2) oral TE in sesame oil (Delatestryl, BTG Pharmaceuticals, Iselin, NJ) at a concentration of 200 mg/ml. A sample size of seven subjects per group was estimated to have an 80% power with an α of 0.05 to detect a 50% in the change in serum T area under the curve between a given dose of T and T plus D or between TE and TE plus D. The oral T in sesame oil was manufactured by the compounding pharmacy at University of Washington. Briefly, micronized T (U.S.P. grade, Spectrum Quality Projects, Gardena, CA) was suspended at 100 mg/ml in sesame oil (N.F. grade, Spectrum Quality Projects) and mixed thoroughly on a magnetic stir plate to create a homogenous T/sesame oil emulsion. The compounding pharmacist then drew up the emulsion into syringes at the desired dose levels (200, 400, and 800 mg) immediately before treatment. The syringe was sent to the Clinical Research Unit, where it was vigorously mixed (by shaking) with milk and administered to the subject. The dose of oral TE in sesame oil was normalized for the T content, so that the subjects in the TE group (molecular weight, 397) were administered 276, 554, and 1108 mg TE, corresponding to 200, 400, and 800 mg T.

The drug exposure period lasted 11 d (Fig. 1). On d 0, subjects received a single injection of the GnRH antagonist acyline (300 μ g/kg, sc), which has been shown to suppress T production in normal men for a minimum of 15 d (29). One, 2, and 3 d after acyline administration, subjects drank 200, 400, or 800 mg T or 276, 554, or 1108 mg TE. Subjects self-administered D (0.5 mg, orally, once daily) on d 5–10 after acyline injection, and doses of T and TE were repeated on days 8, 9, and 10. For safety, subjects

underwent daily testing of liver function (aspartate aminotransferase, bilirubin, and alkaline phosphatase), kidney function (urea nitrogen and creatinine), and hemopoiesis (hemoglobin and hematocrit).

Measurements

After treatment on d 1, 2, 3, 8, 9, and 10, subjects had blood drawn via a heparin-locked iv line at 30 min and 1, 2, 4, 6, 8, 10, 12, and 24 h for measurement of serum T, DHT, E2, and SHBG. Total T was measured by a RIA (Diagnostic Products Corp., Webster, TX). The assay had a sensitivity of 0.35 nmol/liter; interassay variations for low, medium, and high pools of 13.6%, 6.1%, and 6.8%, respectively; and intraassay variations of 10.0%, 5.3%, and 6.6%. The normal range was 8.7–33 nmol/liter. DHT was measured using an RIA kit (Diagnostic Systems Laboratory, Inc., Los Angeles, CA). The sensitivity of this assay was 0.043 nmol/liter, and the intraassay variations for medium and low range pools were 9.9% and 11%, respectively, with interassay coefficients of variations of 19% and 25%. The normal range for serum DHT was 1.0–2.9 nmol/liter. SHBG was measured by RIA (Delphia, Wallac Oy, Turku, Finland). The sensitivity of this assay was 0.2 nmol/liter, and the interassay variations for low, medium, and high pools were 31%, 10.6%, and 6.8%, respectively; the intraassay variations were 3.8%, 1.7%, and 2.2%. The normal range was 3.2–47 nmol/liter. The normal ranges for T, DHT, and SHBG were determined in our laboratory using serum samples obtained from 100 normal men, aged 20–50 yr. Serum E2 was measured in the laboratory of Dr. David Hess (Oregon National Primate Research Center, Portland, OR) with an Elecsys 2010 Platform (Roche, Indianapolis, IN). The sensitivity of this assay was 5.5 pmol/liter, intraassay variations were 3.7%, and 2.8% for medium and high range values, and the interassay coefficient of variation was 4.7%. The normal range for serum E2 in this assay in men was 40–220 pmol/liter.

Statistics

Serum hormone levels at each time point for each dose of T or TE with or without D were compared using a Wilcoxon sign-rank test. Pharmacokinetic parameters between successive doses of T or TE with or without D were compared using a Wilcoxon sign-rank test with a Bonferroni correction for repeated measures (effective α = 0.01). The average concentration during the 24-h period after treatment, the maximum concentration after dosing, time to maximum concentration, area under the curve, and elimination phase half-life ($t_{1/2}$) were calculated using a pharmacokinetics program (PK Solutions, Golden, CO). Statistical analyses were performed using STATA (College Park, TX).

Results

Subjects

Fourteen men were enrolled in the study; seven were randomized to the T group, and seven were randomized to the TE group, but one man assigned to the TE group failed to report for his acyline injection. Therefore, seven men completed the T arm, and six completed the TE arm of the study (Table 1). Except for the subject who failed to appear for his acyline injection, all subjects completed the drug exposure

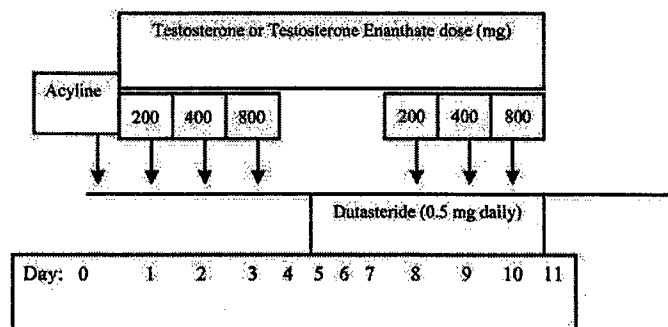


FIG. 1. Study design.

TABLE 1. Baseline characteristics of study subjects by group

| | T group (n = 7) | TE group (n = 6) |
|--------------------------|-----------------|------------------|
| Age (yr) | 24.2 ± 8.7 | 24.7 ± 6.7 |
| Weight (kg) | 77 ± 4.0 | 89 ± 16 |
| Height (cm) | 182 ± 9 | 186 ± 11 |
| BMI (kg/m ²) | 23.3 ± 2.3 | 25.8 ± 4.2 |
| Total T (nmol/liter) | 22.7 ± 8.0 | 17.0 ± 5.8 |
| DHT (nmol/liter) | 1.24 ± 0.46 | 1.1 ± 0.5 |
| SHBG (nmol/liter) | 33.2 ± 9.84 | 24.0 ± 10.7 |
| Free T (pmol/liter) | 435 ± 156 | 341 ± 92 |
| E2 (pmol/liter) | 132 ± 17 | 121 ± 31 |

Values are the mean ± SD. BMI, Body mass index (weight in kilograms/[height in meters]²).

period. There were no serious adverse effects during the study. Nine of the subjects experienced transient mild pruritis at the site of the acyline injection, which resolved in all cases within 1 h of the injection. Eight subjects complained of mild, transient hot flash symptoms toward the end of the study period, presumably due to low T levels; however, no subject complained of feelings of anger, aggression, or irritability during treatment. There were no adverse gastrointestinal symptoms associated with oral T or oral TE in oil. One subject developed a small area of gynecomastia ($<1 \times 1$ cm) immediately under the nipple during the treatment period, but this resolved during follow-up. There were no changes in serum markers of liver or kidney function or in

the hematocrit or hemoglobin during the treatment phase or at follow-up. Furthermore, no significant changes in blood pressure or pulse were observed. T and gonadotropin levels returned to baseline in all subjects during the follow-up period (data not shown). No subjects were lost to follow-up.

Serum T

All subjects were suppressed to castrate levels of T by 24 h after acyline administration (d 0 T, 20.0 ± 7.4 ; d 1 T, 2.3 ± 0.5 nmol/liter; $P < 0.0001$). There was no difference in serum T levels 24 h after acyline between groups [2.3 ± 0.7 (T) vs. 2.3 ± 0.8 (TE); $P = 0.9$]. In addition, mean serum T levels before

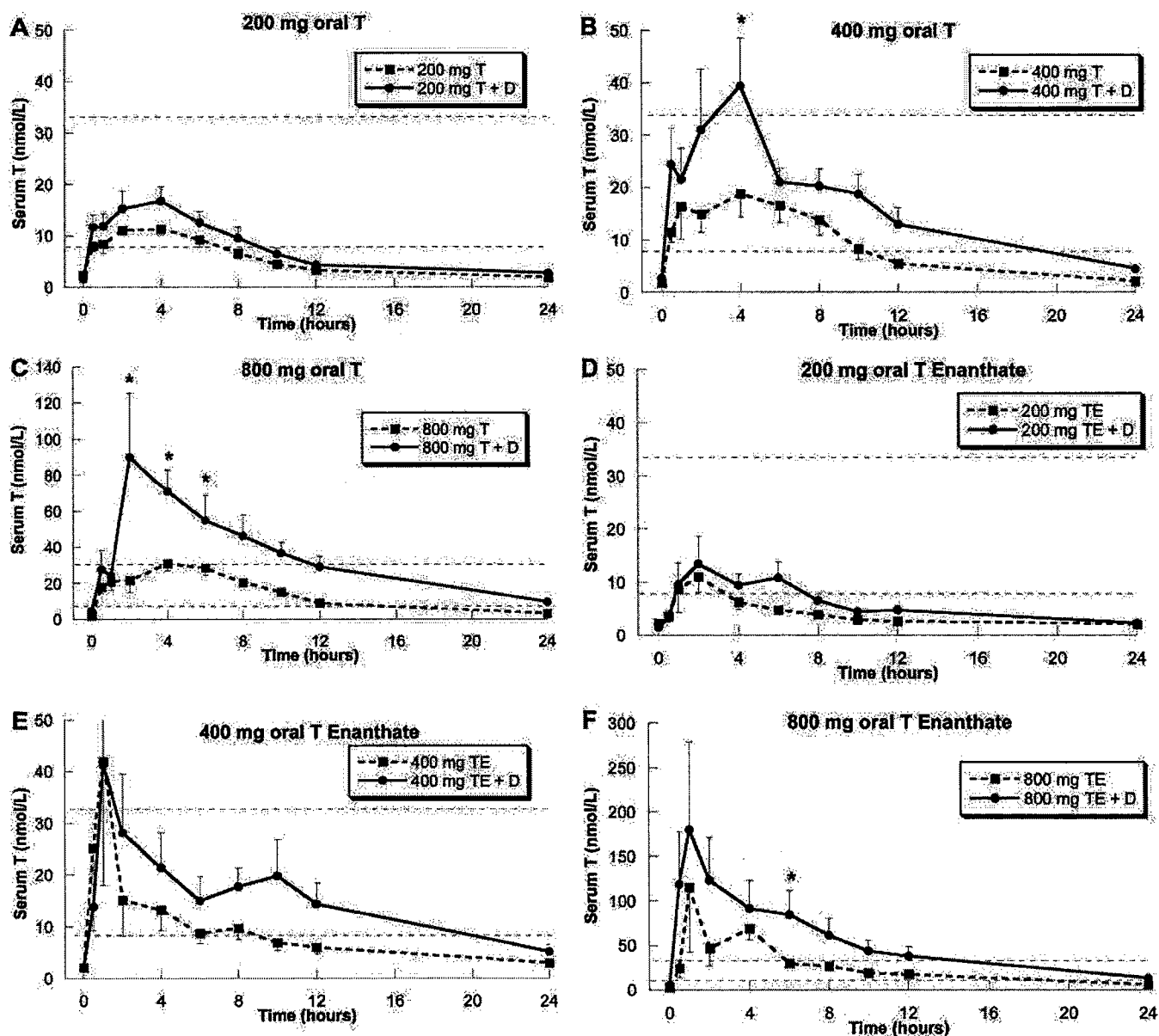


FIG. 2. Serum T concentrations (mean \pm SEM) after oral administration of 200, 400, and 800 mg T in oil (A–C) and TE in oil (D–F) with and without D for 24 h in normal men treated with the GnRH antagonist acyline to temporarily suspend T production. Note the larger y-axis for the 800-mg dose. The dotted lines represent the upper and lower limits of the normal range for serum T. *, $P < 0.05$ compared with T alone.

TABLE 2. T pharmacokinetics after administration of a single dose of oral T and oral TE in oil with and without D to normal men previously administered a GnRH antagonist

| Testosterone (n = 7) | T Only | | | T + D | | |
|----------------------|------------|-----------------------|--------------------------|-------------------------|----------------------------|--------------------------|
| | 200 mg | 400 mg | 800 mg | 200 mg | 400 mg | 800 mg |
| Cmax (nmol/liter) | 12.3 ± 4.1 | 26.1 ± 15.1 | 40.4 ± 10.1 ^a | 22.2 ± 8.4 ^b | 50.3 ± 30.9 ^{a,b} | 122.1 ± 82 |
| Tmax (h) | 2.8 ± 1.9 | 3.9 ± 2.6 | 3.1 ± 2.0 | 3.1 ± 2.0 | 3.8 ± 3.1 | 3.4 ± 1.5 |
| AUC (nmol-h/liter) | 124 ± 28 | 208 ± 74 ^a | 328 ± 72 ^a | 176 ± 46 ^c | 393 ± 140 ^{a,c} | 846 ± 363 ^{a,c} |
| t _{1/2} (h) | 10.4 ± 2.9 | 10.7 ± 6.0 | 8.1 ± 5.0 | 9.9 ± 3.8 | 9.0 ± 2.8 | 7.8 ± 3.2 |

| TE (n = 6) | TE only | | | TE + D | | |
|----------------------|------------|-----------|------------------------|------------|------------------------|-------------|
| | 200 mg | 400 mg | 800 mg | 200 mg | 400 mg | 800 mg |
| Cmax (nmol/liter) | 14.6 ± 8.5 | 51.8 ± 59 | 160.8 ± 149 | 20.2 ± 9.4 | 74 ± 55 ^a | 229 ± 228 |
| Tmax (h) | 3.2 ± 2.6 | 4.1 ± 4.0 | 2.7 ± 1.5 | 4.1 ± 4.2 | 4.3 ± 3.8 | 3.3 ± 2.4 |
| AUC (nmol-h/liter) | 90 ± 27 | 200 ± 140 | 612 ± 249 ^d | 141 ± 41 | 450 ± 196 ^a | 1327 ± 1021 |
| t _{1/2} (h) | 10 ± 2.4 | 10 ± 3.2 | 8.4 ± 3.2 | 9.4 ± 3.2 | 9.2 ± 2.9 | 8.4 ± 2.4 |

Values are the mean ± SD. AUC, Area under the curve; Cmax, maximum concentration after dosing; Tmax, time of maximum concentration.

^a $P < 0.05$ vs. immediately lower dose.

^b $P < 0.05$ vs. T and TE only.

^c $P < 0.01$ vs. T only.

each dose of T were not significantly different from those 24 h after acylone administration.

With the administration of both oral T and oral TE in oil, serum T was significantly increased in a dose-dependent fashion (Fig. 2; $P < 0.01$ for trend). In addition, the maximum concentrations of T, average concentrations of serum T, and area under the curve of serum T increased significantly in a dose-dependent fashion (Table 2 and Fig. 3A), with the maximum concentration of T after oil dosing exceeding the normal range for the 800-mg dose of T and the 400- and 800-mg doses of oral TE in oil. The time of maximum concentration was between 2.5 and 4.5 h in all cases, and the calculated terminal $t_{1/2}$ of oral T and TE in oil was between 7.5 and 11 h.

Coadministration of D with oral T or TE in oil significantly

increased the resulting serum T levels compared with administration of T or TE alone (Fig. 2; $P < 0.01$ for trend). The maximum concentration of T after oral treatment with the combination of T or TE and D exceeded the normal range for both the 400- and 800-mg doses of T and TE in oil. Similar to the administration of T or TE only, the time to maximum concentration remained between 2.5 and 4.5 h, and the calculated terminal $t_{1/2}$ was between 8 and 10 h. The T area under the curve for the combination of T and D was significantly increased at all doses compared with that for T alone [200 mg, 124 ± 28 nmol-h/liter (T alone) vs. 176 ± 45 nmol-h/liter (T + D); 400 mg, 208 ± 74 nmol-h/liter (T alone) vs. 393 nmol-h/liter (T plus D); 800 mg, 328 ± 82 nmol-h/liter (T alone) vs. 846 ± 363 nmol-h/liter (T plus D); $P < 0.01$ for all comparisons].

Serum DHT levels

Serum DHT decreased significantly 24 h after acylone administration (d 0 DHT, 1.6 ± 0.6 nmol/liter; d 1 DHT, 0.6 ± 0.2 nmol/liter; $P < 0.05$). There was no difference in serum DHT levels 24 h after acylone administration between groups (T, 0.5 ± 0.2; TE, 0.6 ± 0.2; $P = 0.63$).

The administration of both oral T and oral TE in oil significantly increased serum DHT in a dose-dependent fashion (Fig. 4). In addition, the maximum concentration of DHT and the area under the curve increased significantly (Table 3), with the maximum concentration of DHT after oral treatment exceeding the normal range for all doses of T and TE in oil. The time of maximum concentration was between 3.9 and 6 h in all cases, and the calculated terminal $t_{1/2}$ of oral T and TE in oil was between 7.5 and 11 h.

Coadministration of D with oral T or TE in oil significantly decreased both maximum and average serum DHT levels compared with the administration of T or TE alone (Fig. 3B and Table 3). The maximum concentration of DHT after oral treatment with the combination of T and D exceeded the normal range at the 800-mg dose of T and at the 400- and 800-mg doses of TE in oil. The time to maximum concentration was between 2.5 and 7.5 h, and the calculated terminal $t_{1/2}$ was between 8 and 10 h. The DHT area under the curve

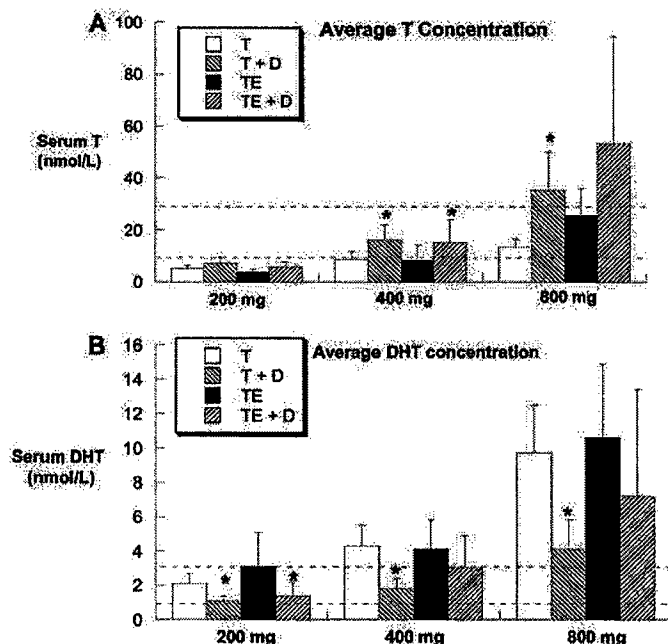


FIG. 3. Average serum T (A) and DHT (B) concentrations (mean ± SD) over the 24-h interval after oral treatment. The dotted lines represent the upper and lower limits of the normal range for serum T. *, $P < 0.05$ compared with T alone.

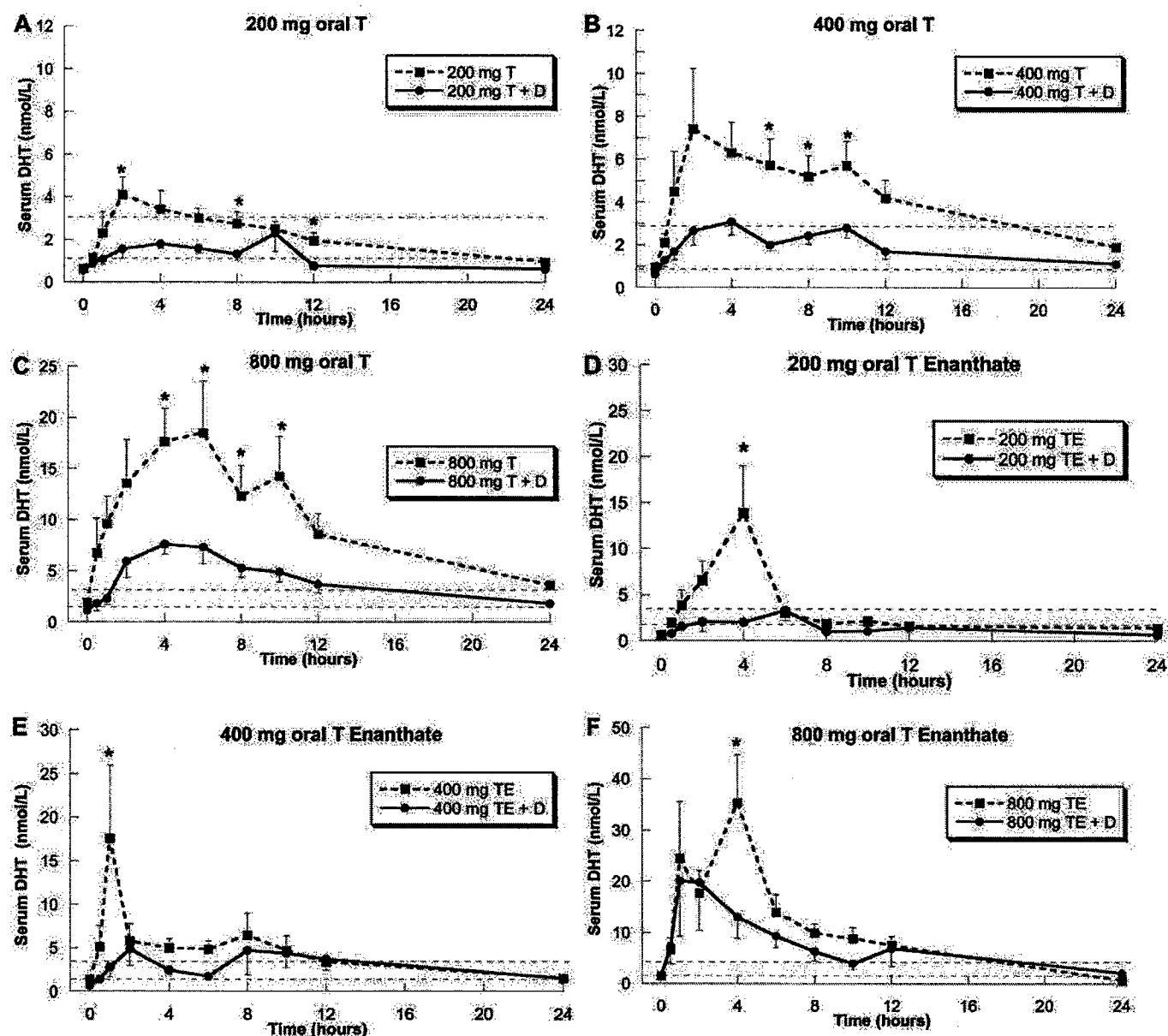


FIG. 4. Serum DHT concentrations (mean \pm SEM) after oral administration of 200, 400, and 800 mg T in oil (A–C) and TE in oil (D–F) with and without D for 24 h in normal men treated with the GnRH antagonist acyline to temporarily suspend T production. Note the larger y-axis for the 800-mg dose. The dotted lines represent the upper and lower limits of the normal range for serum DHT. *, $P < 0.05$ compared with T plus D.

for the combination of T and D was significantly decreased compared with the area under the curve for T alone at all doses.

Serum E2 and SHBG

Mean serum E2 levels were not significantly different between the treatment groups on d 0 [134 ± 21 (T) vs. 116 ± 30 (TE) pmol/liter] or 24 h after acyline administration [94 ± 14 (T) vs. 87 ± 12 (TE) pmol/liter]. With oral administration of T or TE, serum E2 levels increased nonsignificantly compared with baseline levels with the 800-mg dose in both the T and TE groups (Fig. 5), but all E2 levels remained within the normal range. There were no significant differences in

serum E2 between either T or TE alone compared with T or TE with D coadministration. Serum SHBG did not change significantly after administration of acyline or oral administration of T or TE in oil either with or without concomitant D administration (Fig. 6).

Discussion

In this study we have demonstrated that single doses of T or TE when administered orally in oil can result in serum T levels that would be useful for the treatment of T deficiency. Secondly, we have demonstrated that addition of the 5 α -reductase inhibitor D to oral T in oil 1) significantly increases the serum T levels achieved after a given dose of T, and 2)

TABLE 3. DHT pharmacokinetics after administration of a single dose of oral T and oral TE in oil with and without D to normal men previously administered a GnRH antagonist

| T (n = 7) | T Only | | | T + D | | |
|-------------------------------|-----------|-------------------------|-------------------------|------------------------|--------------------------|---------------------------|
| | 200 mg | 400 mg | 800 mg | 200 mg | 400 mg | 800 mg |
| C _{max} (nmol/liter) | 5.6 ± 2.0 | 12.0 ± 3.9 ^a | 30.0 ± 7.0 ^a | 2.2 ± 0.7 ^b | 4.2 ± 1.6 ^{a,b} | 10.3 ± 3.5 ^{a,b} |
| T _{max} (h) | 4.7 ± 3.4 | 5.0 ± 3.8 | 3.9 ± 3.5 | 5.1 ± 3.0 | 6.0 ± 3.3 | 4.6 ± 2.2 |
| AUC (nmol-h/liter) | 51 ± 15 | 106 ± 29 ^a | 239 ± 71 ^a | 25 ± 8.5 ^c | 45 ± 15 ^{a,b} | 99 ± 40 ^{a,b} |
| t _{1/2} (h) | 10 ± 2.3 | 9.3 ± 2.0 | 7.5 ± 3.6 | 9.9 ± 3.8 | 10.6 ± 2.3 | 9.9 ± 2.2 |

| TE (n = 6) | TE only | | | TE + D | | |
|-------------------------------|-----------|-----------|--------------------------|------------------------|------------------------|------------------------|
| | 200 mg | 400 mg | 800 mg | 200 mg | 400 mg | 800 mg |
| C _{max} (nmol/liter) | 15.3 ± 12 | 21.0 ± 19 | 48.8 ± 22.6 ^a | 4.0 ± 2.4 ^c | 8.0 ± 6.5 ^a | 25.3 ± 24 ^c |
| T _{max} (h) | 3.2 ± 1.3 | 4.2 ± 3.5 | 2.5 ± 1.6 | 5.5 ± 3.7 | 7.2 ± 4.0 | 2.7 ± 2.0 |
| AUC (nmol-h/liter) | 75 ± 48 | 100 ± 42 | 253 ± 101 | 35 ± 18 ^c | 72 ± 45 | 173 ± 148 |
| t _{1/2} (h) | 10 ± 2.2 | 9.1 ± 3.5 | 8.6 ± 2.9 | 8.6 ± 3.5 | 8.4 ± 3.4 | 9.0 ± 3.3 |

Values are the mean ± SD. AUC, Area under the curve; C_{avg}, average concentration during 24-h period after dosing; C_{max}, maximum concentration after dosing; T_{max}, time of maximum concentration.

^a *P* < 0.05 vs. immediately lower dose.

^b *P* < 0.01 vs. T only.

^c *P* < 0.05 vs. T and TE only.

attenuates the supraphysiological elevations in serum DHT seen with the administration of oral T or T esters (e.g. TU) without concomitant 5 α -reductase inhibition.

These data contradict the prevailing wisdom in the field, which states that the oral route for T delivery is impractical

due to near-complete hepatic first-pass metabolism of orally administered T (11). Although it is true that the bioavailability of orally administered T is very low, probably around 1% (30, 31), our work demonstrates that if sufficient T is administered orally in oil, potentially therapeutic levels of serum T can be achieved after oral dosing. It is likely that liver metabolism of orally dosed T is extensive, because oral T administered to men with cirrhosis results in serum T levels that are markedly elevated compared with normal controls (32, 33). Whether long-term administration of oral T in oil would induce increased hepatic metabolism of oral T and therefore reduce T bioavailability will be the subject of future research.

Previous studies of the oral administration of T may have found reduced levels of serum T in part due to 5 α -reductase activity in the intestine and liver (34). In this study using T or TE, and in the work of others with TU (24–27), serum levels of DHT after oral administration are markedly elevated, implying that a large fraction of the orally adminis-

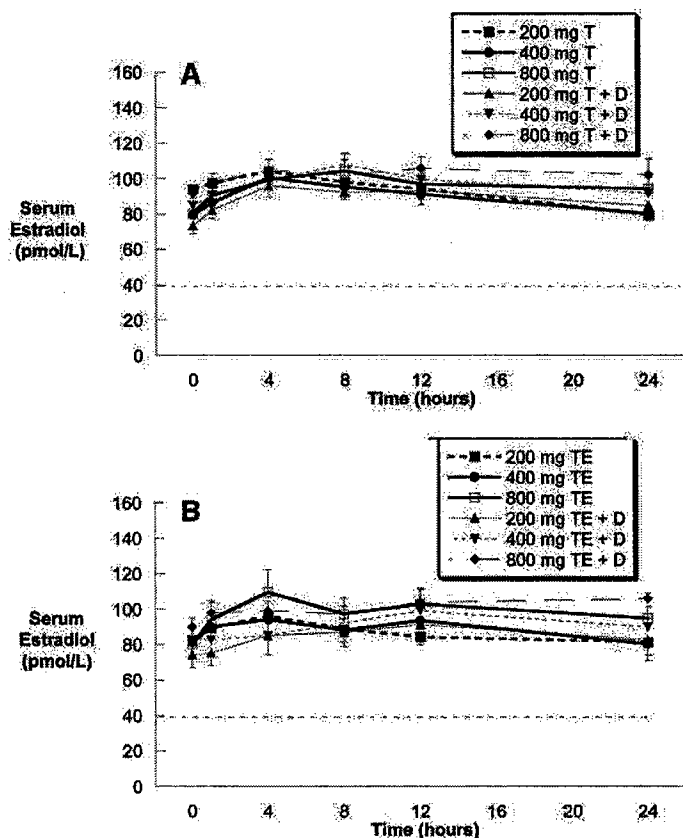


FIG. 5. Serum E2 (mean ± SEM) after oral administration of 200, 400, and 800 mg T (A) and TE (B) in oil with and without D for 24 h in normal men treated with the GnRH antagonist acyline to temporarily suspend T production. The dotted line represents the lower limit of the normal range.

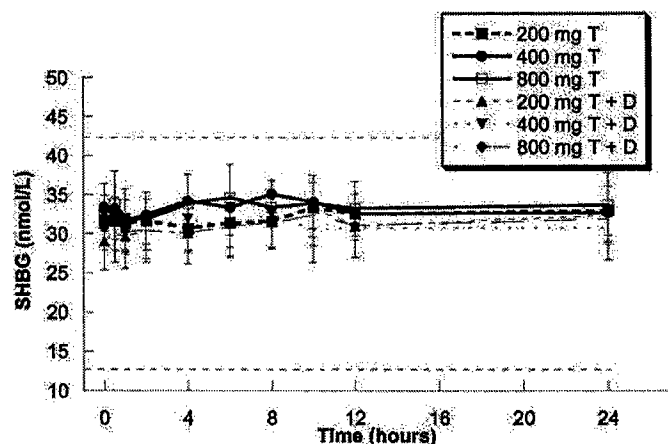


FIG. 6. Serum SHBG concentrations (mean ± SEM) after oral administration of 200, 400, and 800 mg T and TE in oil with and without D for 24 h in normal men treated with the GnRH antagonist acyline to temporarily suspend T production. The dotted lines represent the upper and lower limits of the normal range.

tered T dose may be metabolized in the liver and intestines to DHT. Surprisingly, in this study, the coadministration of a 5 α -reductase inhibitor roughly doubles the average T concentration and the area under the curve for the serum T while reducing the elevations of serum DHT by approximately half. These marked elevations in serum T with concomitant 5 α -reductase inhibition are probably due to inhibition of the 5 α -reductase enzyme in intestine and liver, which appears to account for approximately one half of the metabolism of T after an oral dose. Importantly, the combination of elevated serum T without marked elevations in serum DHT may allow for selective oral androgen therapy, which may be useful in decreasing the risk for DHT-dependent disease, such as benign prostate hyperplasia and prostate cancer.

It is also important to note that previous studies of oral T administration demonstrating poor oral bioavailability of T have used T in powder form at doses of 100 and 200 mg (21–23). We have tested oral T in powder form in doses as high as 400 mg without achieving therapeutic serum T levels (data not shown), implying that the administration of T in oil is crucial for the achievement of the therapeutic serum T levels seen in this study. It has been previously shown that the absorption of oral TU is markedly affected by concomitant intake of fatty foods (27, 30). This is probably due to the fact that much of the orally administered TU is absorbed via the lymphatics (35). In an animal model of TU absorption, more than 80% of the bioavailable T is thought to be absorbed via the lymphatics (36). Whether food intake will affect the absorption of oral T in oil is unknown and probably depends on how much of the dose is absorbed via lymphatics *vs.* via the portal circulation. Because T was administered in oil in this study, some of the dose may have been absorbed via the lymphatics. This might explain in part the unexpectedly long serum half-life of T seen with oral compared with iv administration of T, which has been reported to have a half-life of less than 1 h (31, 37). Another possibility is that there is some degree of enterohepatic circulation of the orally administered T, prolonging the apparent half-life in serum. Because of this uncertainty, the impact of food intake on the absorption and serum levels of T after the administration of oral high dose T will be the subject of future study.

It is important to note that there was no evidence of either liver or kidney toxicity associated with the doses of oral T administered in this study; however, additional long-term study of these doses combined with a 5 α -reductase inhibitor will be required to determine the safety of this approach to T therapy. Although one subject did report transient gynecomastia, this subject's serum E2 level remained within the normal range. Additionally, no subject complained of impotence, decreased libido, or sexual dysfunction during the treatment period. These side effects have been reported when D is administered alone for benign prostate hyperplasia (38); however, in theory, they would be less likely when D is administered in combination with T. Additionally, the implication of long-term 5 α -reductase inhibition will need examination given the increase in high grade prostate cancer (despite an overall decrease in prostate cancer incidence) seen with chronic finasteride administration in the prostate cancer prevention trial (39).

There were slight, nonsignificant increases in serum E2

seen after oral dosing of T and TE in oil. This implies that although orally administered T can undergo aromatization to E2, it does not do so at high levels, suggesting that there is probably little aromatase activity in the intestine and liver in man. This finding is reassuring in showing that orally administered T is likely to allow for the important functions of estrogen in man, such as maintenance of bone density (40), but not lead to an increased risk of estrogen-related side effects such as gynecomastia.

From a practical standpoint, a regimen using oral T in oil in the formulation used in this study may need to be administered twice daily; however, additional refinements of this approach, such as the use of slow-release capsules, may allow for more controlled release of T in the intestine and could lead to a formulation that could be administered orally once daily, a major improvement over current T replacement options.

In conclusion, we have demonstrated that single doses of T or TE, when administered orally in oil, can result in markedly elevated serum levels of T in normal men with induced hypogonadism; such levels would presumably be therapeutically effective in treating testicular failure. In addition, we have demonstrated that addition of the 5 α -reductase inhibitor D to oral T in oil significantly increases the serum T levels observed with a given dose of T and attenuates the supraphysiological elevations in serum DHT seen with the administration of oral T alone. Combinations of oral T and 5 α -reductase inhibitors may allow for an oral, selective form of androgen therapy. Additional studies of the long-term safety, pharmacokinetics, and pharmacodynamics of this combination are warranted to determine whether it might be a clinically useful and attractive method of treating T deficiency.

Acknowledgments

We acknowledge Ms. Amanda Wiseman for assistance with study implementation, and Ms. Dorothy McGuinness for performing the hormone assays.

Received June 25, 2004. Accepted February 9, 2005.

Address all correspondence and requests for reprints to: Dr. John K. Amory, University of Washington, Box 356429, 1959 NE Pacific Street, Seattle, Washington 98195. E-mail: jamory@u.washington.edu.

This work was supported by the National Institute of Child Health and Human Development, a division of the National Institutes of Health, through Cooperative Agreements U54-HD-12629 and U54-HD-42454 as part of the specialized Cooperative Centers Program in Reproductive Research and the Cooperative Contraceptive Research Centers Program. J.K.A. is supported in part by the National Institute of Child Health and Human Development, a division of the National Institutes of Health (NIH), by Grant 1K23-HD-45386-10A1. A portion of this work was conducted through the Clinical Research Center facility at University of Washington and supported by NIH Grant M01-RR-00037.

References

1. Kelch RP, Jenner MR, Weinstein R, Kaplan SL, Grumbach MM 1972 Estradiol and testosterone in the male: secretion by human, simian, and canine testes. *J Clin Invest* 51:824–830
2. Weinstein R, Kelch RP, Jenner MR, Kaplan SL, Grumbach MM 1974 Secretion of androgens and estrogens by the normal and abnormal human testis. *J Clin Invest* 53:1–6
3. Plymate SR 2001 Male hypogonadism. In: Becker KL, ed. *Principles and practice of endocrinology and metabolism*, 3rd Ed. Philadelphia: Lippincott Williams & Wilkins; 1125–1150
4. Katznelson L, Finkelstein JS, Schoenfeld DA, Rosenthal KI, Klibanski A 1996 Increase in bone density and lean body mass during testosterone ad-

- ministration in men with acquired hypogonadism. *J Clin Endocrinol Metab* 81:4358–4365
5. Behre HM, Kliesch S, Leifke R, Link RM, Nieschlag E 1997 Long-term effect of testosterone therapy on bone mineral density in hypogonadal men. *J Clin Endocrinol Metab* 82:2386–2390
 6. Bhasin S, Bremner WJ 1997 Emerging issues in androgen replacement therapy. *J Clin Endocrinol Metab* 1982:3–8
 7. Wang C, Alexander G, Berman N, Salehian B, Davidson T, McDonald V, Steiner B, Hull L, Callegari C, Swerdloff RS 1996 Testosterone replacement therapy improves mood in hypogonadal men—a clinical research center study. *J Clin Endocrinol Metab* 81:3578–3583
 8. Snyder PJ, Peachey H, Berlin JA, Hannoush P, Haddad G, Dlewati A, Santanna J, Loh L, Lenrow DA, Holmes JH, Kapoor SC, Atkinson LE, Strom BL 2000 Effects of testosterone replacement in hypogonadal men. *J Clin Endocrinol Metab* 85:2670–2677
 9. Bagatell CJ, Bremner WJ 1996 Androgens in men—uses and abuses. *N Engl J Med* 334:707–714
 10. Matsumoto AM 2001 Clinical use and abuse of androgens and antiandrogens. In: Becker KL, ed. *Principles and practice of endocrinology and metabolism*, 3rd Ed. Philadelphia: Lippincott Williams & Wilkins; 1181–1199
 11. Snyder P 2001 Androgens. In: Hardman JG, Lumbard LE, eds. *Goodman and Gilman's the pharmacological basis of therapeutics*, 4th Ed. New York: McGraw-Hill; 1635–1648
 12. Westaby D, Ogle SJ, Paradinis FJ, Randell JB, Murray-Lyon IM 1977 Liver damage from long-term methyltestosterone. *Lancet* 2:262–263
 13. Turani H, Levi J, Zevin D, Kessler E 1983 Hepatic lesions in patients on anabolic androgenic therapy. *Isr J Med Sci* 19:332–337
 14. Lowdell CP, Murray-Lyon IM 1985 Reversal of liver damage due to long term methyltestosterone and safety of non-17- α alkylated androgens. *Br Med J* 291:637–645
 15. Cabasso A 1994 Peliosis hepatitis in a young adult bodybuilder. *Med Sci Sports Exerc* 26:2–4
 16. Pavlatos AM, Fultz O, Monberg MJ, Vootkur A 2001 Review of oxymetholone: a 17- α -alkylated anabolic-androgenic steroid. *Clin Ther* 23:789–801
 17. Fossa SD, Opjordsmoen S, Haug E 1999 Androgen replacement and quality of life in patients treated for bilateral testicular cancer. *Eur J Cancer* 35:1220–1225
 18. Amory JK, Matsumoto AM 1998 The therapeutic potential of testosterone patches. *Exp Opin Invest Drugs* 7:1977–1985
 19. Swerdloff RS, Wang C, Cunningham G, Dobs A, Iranmanesh A, Matsumoto AM, Snyder PJ, Weber T, Longstreth J, Berman N 2000 Long-term pharmacokinetics of transdermal testosterone gel in hypogonadal men. *J Clin Endocrinol Metab* 85:4500–4507
 20. Foss GL 1939 Clinical administration of androgens. *Lancet* 1:502–504
 21. Nieschlag E, Mauss J, Coert A, Kicovic P 1975 Plasma androgen levels in men after oral administration of testosterone and testosterone undecanoate. *Acta Endocrinol (Copenh)* 79:366–374
 22. Johnsen SG, Bennett EP, Jensen VG 1974 Therapeutic effectiveness of oral testosterone. *Lancet* 2:1473–1475
 23. Daggett PR, Wheeler MJ, Nabarro JDN 1978 Oral testosterone, a reappraisal. *Horm Res* 9:121–129
 24. Skakkebaek NE, Bancroft J, Davidson DW, Warner P 1981 Androgen replacement with oral testosterone undecanoate in hypogonadal men: a double-blind controlled study. *Clin Endocrinol (Oxf)* 14:49–61
 25. Gooren LJG 1994 A ten-year safety study of the oral androgen testosterone undecanoate. *J Androl* 15:212–215
 26. Houwing NS, Maris F, Schnabel PG, Bagchus WM 2003 Pharmacokinetic study in women of three different doses of a new formulation of oral testosterone undecanoate, Andriol Testocaps. *Pharmacotherapy* 23:1257–1265
 27. Bagchus WM, Hust R, Maris F, Schnabel PG, Houwing NS 2003 Important effect of food on the bioavailability of oral testosterone undecanoate. *Pharmacotherapy* 23:319–325
 28. Roehrborn CG, Boyle P, Nickel JC, Hoefner K, Andriole G 2002 Efficacy and safety of a dual inhibitor of 5 α -reductase types 1 and 2 (dutasteride) in men with benign prostatic hyperplasia. *Urology* 60:434–441
 29. Herbst KL, Coviello AD, Page S, Amory JK, Anawalt BD, Bremner WJ 2004 A single dose of the potent gonadotropin-releasing hormone antagonist acyl-line suppresses gonadotropins and testosterone for 2 weeks in healthy young men. *J Clin Endocrinol Metab* 89:5959–5965
 30. Frey H, Aakvaag A, Saanum D, Falch J 1979 Bioavailability of oral testosterone in males. *Eur J Clin Pharmacol* 16:345–349
 31. Tauber U, Schroder K, Dusterberg B, Matthes H 1986 Absolute bioavailability of testosterone after oral administration of testosterone undecanoate and testosterone. *Eur J Drug Metab Pharmacokinet* 11:145–149
 32. Glud C, Bennett P, Dietrichson O, Johnsen SG, Ranek L, Swendsen LB, Juhl E 1981 Short-term parenteral and peroral testosterone administration in men with alcoholic cirrhosis. *Scand J Gastroenterol* 16:749–755
 33. Glud C, Bahnsen M, Bennett P, Dietrichson O, Henriksen JH, Johnsen SG, Swendsen LB, Brodthagen UA, Juhl E 1983 Oral testosterone load related to liver function in men with alcoholic cirrhosis. *Scand J Gastroenterol* 18:391–396
 34. Thigpen AE, Silver RJ, Guileyardo JM, Casey ML, McConnell JD, Russell DW 1993 Tissue distribution and ontogeny of steroid 5 α reductase isozymes expression. *J Clin Invest* 92:903–910
 35. Horst HJ, Holtje WJ, Dennis M, Coert A, Geelen J, Voigt KD 1976 Lymphatic absorption and metabolism of orally administered testosterone undecanoate in man. *Klin Wschr* 54:875–879
 36. Shackelford DM, Faassen WA, Houwing N, Lass H, Edwards GA, Porter CJ, Charman WN 2003 Contribution of lymphatically transported testosterone undecanoate to the systemic exposure of testosterone after oral administration of two andriol formulations in conscious lymph duct-cannulated dogs. *J Pharmacol Exp Ther* 306:925–933
 37. Southren AK, Gordon GG, Tochimoto S, Pinzon G, Lane DR, Stypulkowski W 1967 Mean plasma concentration, metabolic clearance and basal testosterone production rates of testosterone in normal young men and women using a constant infusion procedure: effect of time of day and plasma concentration on the metabolic clearance rate of testosterone. *J Clin Endocrinol* 27:686–694
 38. Andriole GL, Kirby R 2003 Safety and tolerability of the dual 5- α -reductase inhibitor dutasteride in the treatment of benign prostatic hyperplasia. *Eur Urol* 44:82–88
 39. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, Minasian LM, Ford LG, Lippman SM, Crawford ED, Crowley JJ, Coltman Jr CA 2003 The influence of finasteride on the development of prostate cancer. *N Engl J Med* 349:215–224
 40. Khosla S, Melton LJ, Riggs BL 2001 Estrogens and bone health in men. *Calcif Tissue Int* 69:189–192

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

Exogenous Testosterone or Testosterone with Finasteride Increases Bone Mineral Density in Older Men with Low Serum Testosterone

JOHN K. AMORY, NELSON B. WATTS, KIRK A. EASLEY, PAUL R. SUTTON,
BRADLEY D. ANAWALT, ALVIN M. MATSUMOTO, WILLIAM J. BREMNER, AND J. LISA TENOVER

Departments of Medicine (J.L.T.) and Biostatistics (K.A.E.), Emory University School of Medicine, Rollins School of Public Health, Atlanta, Georgia 30329; Department of Medicine (N.B.W.), University of Cincinnati College of Medicine, Cincinnati, Ohio 45267; and Department of Medicine (J.K.A., P.R.S., B.D.A., A.M.M., W.J.B.), Veterans Affairs-Puget Sound Health Care System (B.D.A. and A.M.M.), and Geriatric Research, Education and Clinical Center (A.M.M.), University of Washington School of Medicine, Seattle, Washington 98195

Older men, particularly those with low serum testosterone (T) levels, might benefit from T therapy to improve bone mineral density (BMD) and reduce fracture risk. Concerns exist, however, about the impact of T therapy on the prostate in older men. We hypothesized that the combination of T and finasteride (F), a 5 α -reductase inhibitor, might increase BMD in older men without adverse effects on the prostate. Seventy men aged 65 yr or older, with a serum T less than 12.1 nmol/liter on two occasions, were randomly assigned to receive one of three regimens for 36 months: T enanthate, 200 mg im every 2 wk with placebo pills daily (T-only); T enanthate, 200 mg every 2 wk with 5 mg F daily (T+F); or placebo injections and pills (placebo). Low BMD was not an inclusion criterion. We obtained serial measurements of BMD of the lumbar spine and hip by dual x-ray absorptiometry. Prostate-specific antigen (PSA) and prostate size were measured at baseline and during treatment to assess the impact of therapy on the prostate. Fifty men completed the 36-month protocol. By an intent-to-treat analysis including all men for as long as they contributed data, T therapy for 36 months increased BMD in these men at the lumbar spine [$10.2 \pm 1.4\%$ (mean percentage increase from baseline \pm SEM; T-only) and $9.3 \pm 1.4\%$ (T+F) vs. $1.3 \pm 1.4\%$ for placebo ($P < 0.001$)] and in the hip [$2.7 \pm 0.7\%$ (T-only) and $2.2 \pm 0.7\%$ (T+F) vs. $-0.2 \pm 0.7\%$ for placebo, ($P \leq 0.02$)]. Significant

increases in BMD were seen also in the intertrochanteric and trochanteric regions of the hip. After 6 months of therapy, urinary deoxypyridinoline (a bone-resorption marker) decreased significantly compared with baseline in both the T-only and T+F groups ($P < 0.001$) but was not significantly reduced compared with the placebo group. Over 36 months, PSA increased significantly from baseline in the T-only group ($P < 0.001$). Prostate volume increased in all groups during the 36-month treatment period, but this increase was significantly less in the T+F group compared with both the T-only and placebo groups ($P = 0.02$). These results demonstrate that T therapy in older men with low serum T increases vertebral and hip BMD over 36 months, both when administered alone and when combined with F. This finding suggests that dihydrotestosterone is not essential for the beneficial effects of T on BMD in men. In addition, the concomitant administration of F with T appears to attenuate the impact of T therapy on prostate size and PSA and might reduce the chance of benign prostatic hypertrophy or other prostate-related complications in older men on T therapy. These findings have important implications for the prevention and treatment of osteoporosis in older men with low T levels. (*J Clin Endocrinol Metab* 89: 503–510, 2004)

TWENTY PERCENT OF men over age 60 have serum testosterone (T) concentrations below the normal range for young men (1, 2). Because low T levels are associated with an increased risk of osteoporosis and fracture (3–7), T therapy in older men might increase bone mineral density (BMD) and reduce fracture risk. Studies in young, hypogonadal men have demonstrated that T therapy increases BMD (8–11), but few studies have investigated older patients, who are at greater risk of fracture. Two randomized clinical trials of transdermal T treatment in men over the age of 64 yr have been published (12, 13). In the first study, vertebral but not hip BMD increased, and only in those with low pretreatment

T levels (12). In the second study, T prevented the loss of hip BMD observed in the placebo-treated men (13). Therefore, significant questions still exist about the ability of T therapy in older men to have significant impact on bone health.

The relative roles of T and its metabolite, dihydrotestosterone (DHT), in regulating BMD are not clear. Because DHT contributes to the development of benign prostatic hypertrophy (BPH) and possibly prostate cancer, increasing T levels without also increasing DHT might be preferable in older men, especially if DHT has little or no effect on BMD. Finasteride (F) inhibits DHT production by blocking the enzyme 5 α -reductase, which converts T to DHT, and has been used safely to treat BPH in older men without compromising BMD (14–16).

We hypothesized that long-term im T therapy in older men who had serum T below the range of normal for young adult men would significantly increase BMD. Furthermore, we hypothesized that the addition of the 5 α -reductase inhibitor F would have no impact on T-mediated increases in BMD but

Abbreviations: BMD, Bone mineral density; BPH, benign prostatic hypertrophy; CV, coefficient of variation; DHT, dihydrotestosterone; E2, estradiol; F, finasteride; po, per os; PSA, prostate-specific antigen; T, testosterone; TE, T enanthate.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

would minimize the potential for adverse effects on prostate health. Therefore, we conducted a randomized, double-blind, placebo-controlled trial of im T administration, with or without F, to test these hypotheses.

Subjects and Methods

Subjects

Men aged 65 yr and older were recruited using advertisements and direct mailings. The inclusion criterion was a nonfasting, morning serum total T level below 12.1 nmol/liter (350 ng/dl) for 2 d. Exclusion criteria included the following: severe illness; use of medications including anabolic steroids, antiandrogens, glucocorticoids, bisphosphonates, diuretics, calcitonin, seizure medications, or warfarin; Paget's disease; smoking or heavy alcohol use; sleep apnea; hematocrit greater than 48%; total cholesterol above 300 mg/dl; abnormal kidney, liver, thyroid, adrenal, or pituitary function; regular exercise more than three times a week; prostate issues [prostate cancer, a prostate nodule on exam, prostate-specific antigen (PSA) >4.0 ng/ml, or International Prostate Symptom Score >8]; urinary postvoid residual by ultrasound of more than 149 ml; or an abnormal transrectal ultrasound. Reduced BMD was not an inclusion criterion. The Institutional Review Board of Emory University, where all subject interactions occurred, approved the study, and subjects gave written informed consent before screening.

A total of 676 men were evaluated for eligibility. Of these, 283 men were potentially eligible and underwent T measurement. One hundred ten men met the T criterion and underwent further screening tests; 70 men were enrolled. Forty men who passed initial screening were not enrolled for the following reasons: abnormal PSA, prostate ultrasound, postvoid residual, or symptom score (11); pituitary, thyroid, or adrenal disease (5); medical illness (4); second T levels above 350 ng/dl (4); total cholesterol above 300 mg/dl (1); or being eligible but refusing enrollment (15).

Study design

Participants were randomized to one of three treatment groups: 1) T-only group, T enanthate (TE; Schein Pharmaceuticals, Florham Park, NJ) 200 mg im every 2 wk, plus placebo pill orally [*per os* (po)] daily; 2) T+F group, TE 200 mg im every 2 wk, plus F (Merck & Co., Rahway, NJ) 5 mg po daily; or 3) placebo group, sesame oil placebo 1 ml im every 2 wk, plus placebo pill po daily. The estimate of sample size for the trial was based on the percentage change in BMD from baseline to 6-month follow-up. Assuming a clinically important increase on average of 1% in the T+F group, no change on average in the placebo group, and an estimated SD in each group of 1%, a sample size of 17 men per group ensured approximately 80% statistical power to detect a treatment difference of 1% (significance level, 0.05; two-sided test) if the true difference between groups was a 1% BMD increase from baseline to 6-month follow-up. Allowing for a 30% dropout rate over 3 yr, 70 patients were randomized in the trial.

The order of treatment assignment was randomly computer-generated in permuted blocks of six. Participants were treated for 36 months. Only the research pharmacist and safety monitoring board knew of the randomization. A nurse administered the injections, and 98% occurred within 2 d of the scheduled time. There was 95% compliance with the daily F or placebo in the enrolled subjects based on monthly pill counts. The study design included the potential for dose reduction of T or placebo injection by decrements of 0.2 ml (40 mg of TE for subjects actually receiving T) for a hematocrit of more than 52% on safety monitoring performed at 2, 4, 8, 12, 18, 24, and 30 months. Calcium and vitamin D supplements were not provided, but patients were allowed to continue these medications if they were taking them already. Participants were queried at the beginning and end of the study in regard to the intake of these supplements, with no significant change in their use being noted. Specifically, two men in the placebo group, one man in the T-only group, and none in the T+F group were taking calcium supplements during the study. No subject was taking additional vitamin D.

For men who discontinued the study prematurely, telephone follow-up was conducted to ascertain clinical outcomes.

Measurements

At baseline and after 6, 12, 18, 24, and 36 months of treatment, BMD was measured at the lumbar spine (L1-L4; anteroposterior view only) and in the nondominant hip by dual x-ray absorptiometry using a Hologic QDR-2000 densitometer (Hologic, Waltham MA) that was standardized daily. The intraperson coefficient of variation (CV) was 1.0% for both the spine and the hip. T and Z scores were calculated using male databases; the manufacturer's database was used for the spine, and the National Health and Nutrition Examination Survey III was used for all of the hip measurements. One of the investigators (N.B.W.) who was blinded to treatment analyzed all of the BMD measurements and excluded from analysis vertebrae that showed localized degenerative change, compression fractures, or other confounding factors. One or two vertebrae were deleted from analysis if there was obvious degenerative change on the image and/or if it was 1 SD or more higher than the lowest vertebrae (six placebo subjects, five T-only subjects, and five T+F subjects). If three or more vertebrae showed evidence of degenerative change, the spine measurement was considered invalid and was not used (no placebo subjects, two T-only subjects, and one T+F subject).

Blood was drawn for hormone measurements in the morning at baseline and immediately before injections after 2, 4, 6, 8, 12, 18, 24, 30, and 36 months of treatment. Samples at baseline 6, 12, 18, 24, and 36 months were fasting samples, whereas the other samples were nonfasting. Blood was drawn for markers of bone metabolism after a 12-h fast at baseline and after 6 months of treatment. For a subset of men ($n = 22$), additional morning blood was drawn at the end of the first study year on d 3 or 4, 7, and 11 of the T-dosing period to obtain between-nadir samples. Serum samples for 25-hydroxyvitamin D and intact PTH were assayed immediately. All other samples were stored frozen at -70°C until the end of the study, when serum samples from each participant were assayed concurrently. A 2-h morning urine was collected for measurement of deoxypyridinoline at baseline and after 6 months of treatment. T, SHBG, and estradiol (E2) were measured using fluoroimmunoassays (Delfia, Wallac Oy, Turku, Finland). The intraassay and interassay CVs for midrange measurements were 4.5 and 9.5% for T, 4.0 and 11.1% for SHBG, and 3.6 and 6.0% for E2. The normal range is 12–33 nmol/liter for T and 60–220 pmol/liter for E2. DHT was measured by RIA (Endocrine Sciences, Calabasas Hills, CA); the midrange intraassay and interassay CVs were 6.6 and 14%, respectively. Non-SHBG-bound, bioavailable T was assayed using RIA after ammonium sulfate precipitation [Centre Hospitalier de l'Université at Laval University (CHUL) Research Center, Sainte-Foy, Quebec, Canada]; the midrange intraassay and interassay CVs were 7.4 and 12%, respectively. Osteocalcin was measured by RIA (Diagnostic Systems Laboratories, Inc., Webster, TX); the midrange intraassay and interassay CVs were both 7.2%. Bone-specific alkaline phosphatase was measured by immunoassay (Metra Biosystems, Mountain View, CA); the midrange intraassay and interassay CVs were 1.4 and 4.8%, respectively. Urinary deoxypyridinoline was measured by chromatography after acid hydrolysis and was normalized to urinary creatinine; midrange intraassay and interassay CVs were 8 and 15%, respectively. Intact PTH was measured by a chemiluminescent assay (Diagnostic Products Corp., Los Angeles, CA); midrange intraassay and interassay CVs were 5.1 and 5.3%, respectively.

Participant monitoring

Participants were examined monthly. Measurements of hematocrit and transaminases occurred every 2 months for 1 yr, and every 6 months thereafter. Prostate volume was assessed by transrectal ultrasound (Brüel & Kjaer, Borås, Sweden) at baseline (model 3535) and at the end of treatment (model 1846 PM) using established techniques (17, 18). PSA levels were measured every 4 months during the first year and every 6 months thereafter; digital rectal examination was performed every 6 months.

Statistical analysis

The primary analyses of the data were performed according to patients' original treatment assignment (*i.e.* intention-to-treat analyses), and all men were included in the analyses for as long as they contributed data. Baseline characteristics between treatment groups were compared with the Kruskal-Wallis test. Repeated-measures analyses for each of the

four BMD measurements were analyzed as percentage change from baseline with a means model with SAS Proc Mixed (version 8; SAS Institute, Inc., Cary, NC) providing separate estimates of the means by time on the study (6, 12, 18, 24, 30, and 36 months) and treatment groups. An unstructured variance-covariance form among the repeated measurements was assumed for each outcome, and estimates of the SE values of parameters were used to perform statistical tests and construct 95% confidence intervals. Student's *t* tests were used to compare the pairwise differences between the model-based treatment means (least-squares means) at each time point or treatment month. The model-based means are unbiased with unbalanced and missing data, as long as the missing data are noninformative (missing at random). A dropout process is assumed to be missing at random if, depending on the observed data, the dropout is independent of the unobserved measurements. Mean changes over time within a treatment group were tested for linear trend. Repeated-measures analyses were also performed for T, DHT, E2, and PSA after a log transformation, and for prostate volume, hematocrit, hemoglobin, and lipids. The Wilcoxon signed-rank test was used to compare change from baseline to 6 months within each treatment group for six markers of bone metabolism. Statistical tests were two-sided. A Bonferroni adjustment ($P < 0.0167$) was used for the three pairwise comparisons performed at each treatment month.

Results

Seventy men, with a mean age of 71 ± 4 yr (range, 65–83 yr), participated in the study. Twenty-four were randomized to T-only, 22 to T+F, and 24 to placebo. Fifty men completed the entire 36 months of the study. Of the 20 men who did not complete the study, six were in the placebo group, and seven each were in the T-only and T+F groups. Reasons for discontinuation included the following: personal reasons (10 men), intercurrent illness (7 men), or a new diagnosis of

prostate cancer (3 men). At baseline, the three treatment groups did not differ significantly from each other in age, body mass index, hormone levels, BMD, prostate volume, or PSA (Table 1). Twenty-four of the 70 men (10 in the placebo group, 8 in the T-only, and 6 in the T+F group) had baseline serum E2 levels that were below the lower limit of normal. The baseline BMD for the hip and lumbar spine for the participants was similar to that for a standard male population of the same age (Z scores, Table 1). A total of seven men, two each in the placebo and T+F groups and three in the T-only group, had low baseline lumbar-spine BMD (T score more than 2.5 sd below peak bone mass for young men), whereas four men, two in the placebo group and one each in the T-only and T+F groups, had low baseline hip BMD. There were no significant differences in the baseline characteristics between the men who discontinued and those who completed the study. Reduction of T dosage was necessary in 14 men (seven in the T-only group and seven in the T+F group *vs.* none in the placebo group). After the decrease in T dosage, the final mean (\pm sd) dose of TE was 158 ± 36 mg for the T-only group and 164 ± 40 mg for the T+F group every 2 wk.

Mean nadir serum total T, bioavailable T, and E2 levels in the T-only and T+F groups significantly increased throughout the treatment period (Fig. 1, A–C), whereas these hormone levels did not change in the placebo group. Nadir serum total T and E2 levels tended to be higher in the T+F group compared with the T-only group, but this difference

TABLE 1. Baseline characteristics (mean \pm SD) of 70 older men administered im T alone, T with F, or placebo for 36 months

| Characteristic | Placebo (n = 24) | T-only group (n = 24) | T + F group (n = 22) | P |
|--------------------------------------|------------------|-----------------------|----------------------|------|
| Age (yr) | 71 ± 5 | 71 ± 4 | 71 ± 4 | 0.99 |
| Body mass index (kg/m ²) | 27.8 ± 3.6 | 28.7 ± 3.6 | 27.0 ± 2.7 | 0.24 |
| Hormones | | | | |
| Total testosterone (nmol/liter) | 10.5 ± 1.7 | 9.9 ± 1.6 | 10.1 ± 2.1 | 0.36 |
| Dihydrotestosterone (nmol/liter) | 1.0 ± 0.5 | 0.8 ± 0.3 | 0.9 ± 0.2 | 0.48 |
| Estradiol (pmol/liter) | 83.3 ± 44.4 | 71.5 ± 33.7 | 84.0 ± 33.3 | 0.47 |
| SHBG (nmol/liter) | 44.0 ± 18.1 | 45.2 ± 16.6 | 48.2 ± 15.0 | 0.55 |
| Bioavailable T (nmol/liter) | 3.5 ± 1.3 | 3.3 ± 1.2 | 3.4 ± 1.2 | 0.62 |
| BMD | | | | |
| Lumbar spine | | | | |
| Density (g/cm ²) | 1.04 ± 0.15 | 1.06 ± 0.16 | 1.03 ± 0.19 | 0.81 |
| T score ^a | -0.48 ± 1.47 | -0.30 ± 1.56 | -0.53 ± 1.66 | |
| Z score ^a | 0.44 ± 1.49 | 0.69 ± 1.62 | 0.51 ± 1.67 | |
| Total hip | | | | |
| Density (g/cm ²) | 0.96 ± 0.13 | 0.96 ± 0.14 | 0.90 ± 0.23 | 0.67 |
| T score | -0.87 ± 0.89 | -0.84 ± 1.13 | -1.02 ± 0.80 | |
| Z score | 0.30 ± 0.86 | 0.24 ± 1.21 | 0.12 ± 0.79 | |
| Intertrochanter | | | | |
| Density (g/cm ²) | 1.11 ± 0.15 | 1.11 ± 0.17 | 1.11 ± 0.13 | 0.96 |
| T score | -0.90 ± 0.88 | -0.77 ± 1.17 | -0.80 ± 0.96 | |
| Z score | 0.06 ± 1.10 | 0.29 ± 1.21 | 0.16 ± 0.82 | |
| Trochanter | | | | |
| Density (g/cm ²) | 0.74 ± 0.11 | 0.75 ± 0.11 | 0.71 ± 0.10 | 0.29 |
| T score | -0.92 ± 2.32 | -0.39 ± 1.10 | -0.73 ± 0.86 | |
| Z score | 0.27 ± 1.16 | 0.45 ± 1.17 | 0.13 ± 0.85 | |
| Femoral neck | | | | |
| Density (g/cm ²) | 0.81 ± 0.12 | 0.78 ± 0.13 | 0.74 ± 0.09 | 0.19 |
| T score | -1.56 ± 1.07 | -1.77 ± 1.16 | -2.03 ± 0.87 | |
| Z score | 0.42 ± 1.06 | 0.19 ± 1.17 | 0.03 ± 0.86 | |
| Prostate parameters | | | | |
| PSA (ng/dl) | 1.4 ± 1.1 | 0.9 ± 0.8 | 1.0 ± 0.6 | 0.08 |
| Prostate volume (cm ³) | 32 ± 14 | 29 ± 11 | 33 ± 16 | 0.80 |

^a T score compares the BMD to the mean for young normal males and Z score compares it with age-matched controls.

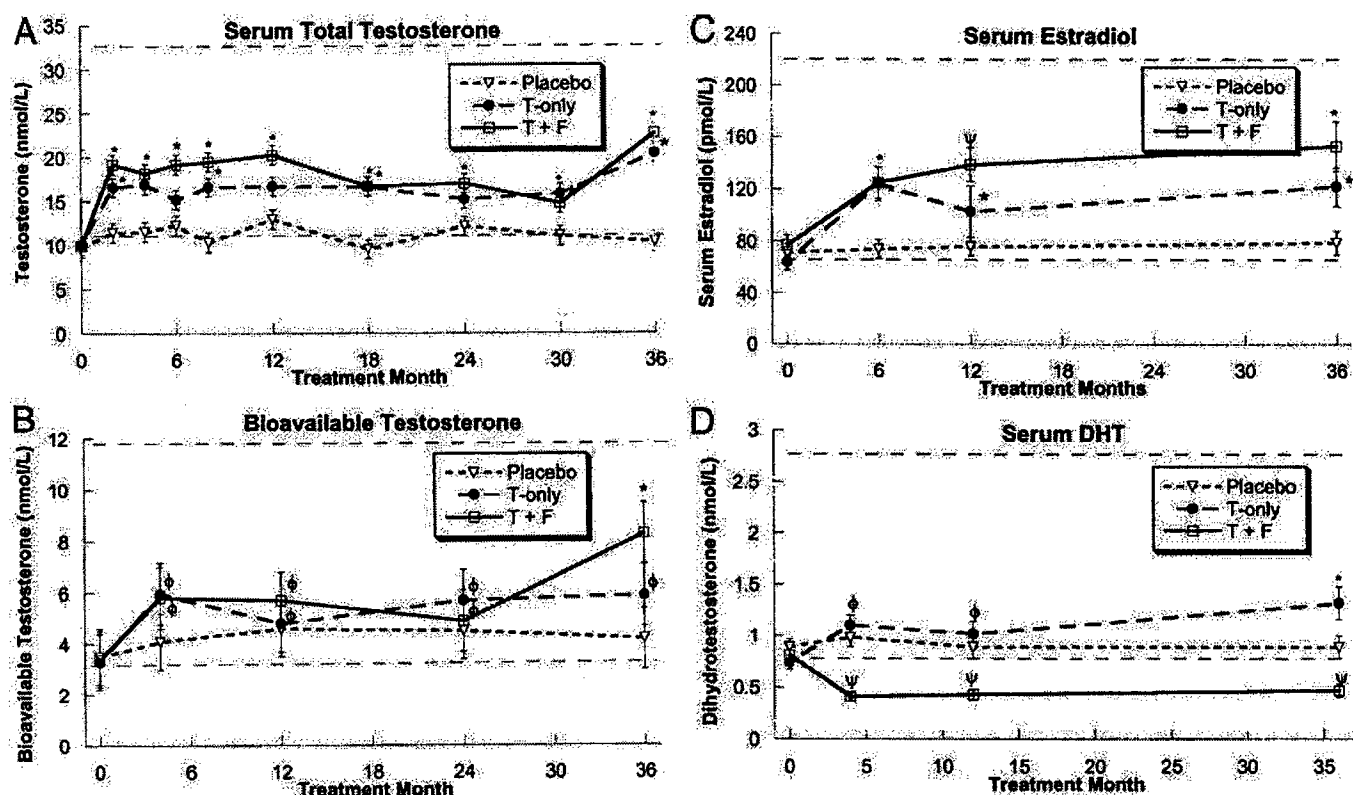


FIG. 1. Geometric mean (\pm SEM) nadir serum total T (A), bioavailable (non-SHBG-bound) T (B), E2 (C), and DHT (D) in older men with low T who were treated with either T (T-only), T and F (T+F), or placebo for 36 months. Horizontal dotted lines represent normal ranges. *, $P < 0.05$ compared with baseline and placebo; ψ , $P < 0.05$ compared with baseline, placebo, and T-only; ϕ , $P < 0.05$ compared with baseline.

did not reach statistical significance for any time point for total T and was only significant at month 12 for E2 ($P = 0.03$). For the subset of men from whom blood was sampled at multiple times throughout the 2-wk T-dosing period (six men in the placebo group, seven men in the T-only group, and nine men in the T+F group), peak serum total T levels were at or above the normal serum T range for the two T treatment groups, with a mean peak value for the T-only group being 35.9 ± 12.1 nmol/liter (mean \pm SD) and that for the T+F group being 43.5 ± 7.6 nmol/liter. Average total T levels during the 2-wk dosing interval were 25.8 ± 6.0 , 33.0 ± 6.4 , and 11.8 ± 2.3 nmol/liter for the T-only, T+F, and placebo groups, respectively. In comparing these two T treatment groups, there was no difference in peak T levels ($P = 0.13$), but the average total T levels were somewhat higher in the T+F group ($P = 0.04$).

Mean nadir serum DHT levels did not change throughout the study in the placebo group, increased significantly in the T-only group, and decreased in the T+F group ($P < 0.001$ compared with baseline and placebo) by 6 months and remained suppressed throughout treatment (Fig. 1D). The maximum decline in serum DHT levels in the T+F group was 50% below baseline, which was reached at treatment month 4.

BMD and metabolism

BMD of the lumbar spine, total hip, and trochanteric and intertrochanteric regions increased in both the T-only and the

T+F groups during the study period, whereas those in the placebo group did not change ($P < 0.001$; Table 2 and Fig. 2, A–D). The mean percentage increase from baseline in BMD of the lumbar spine was significant ($P < 0.001$) for the T-only and T+F groups, but the mean did not change for men in the placebo group ($P = 0.39$ for linear trend). There was no significant change over the 36 months in the BMD at the femoral neck in any of three treatment groups ($P = 0.16$). In the T groups, increases in lumbar BMD were positively correlated with magnitude of increase in both serum total T ($r = 0.44$; $P = 0.001$), bioavailable T ($r = 0.45$ and $P = 0.009$), and serum E2 ($r = 0.45$; $P = 0.0006$) but were not related to baseline BMD; to baseline levels of total T, bioavailable T, DHT, or E2; or to baseline levels of T or E2 after correction for baseline SHBG levels.

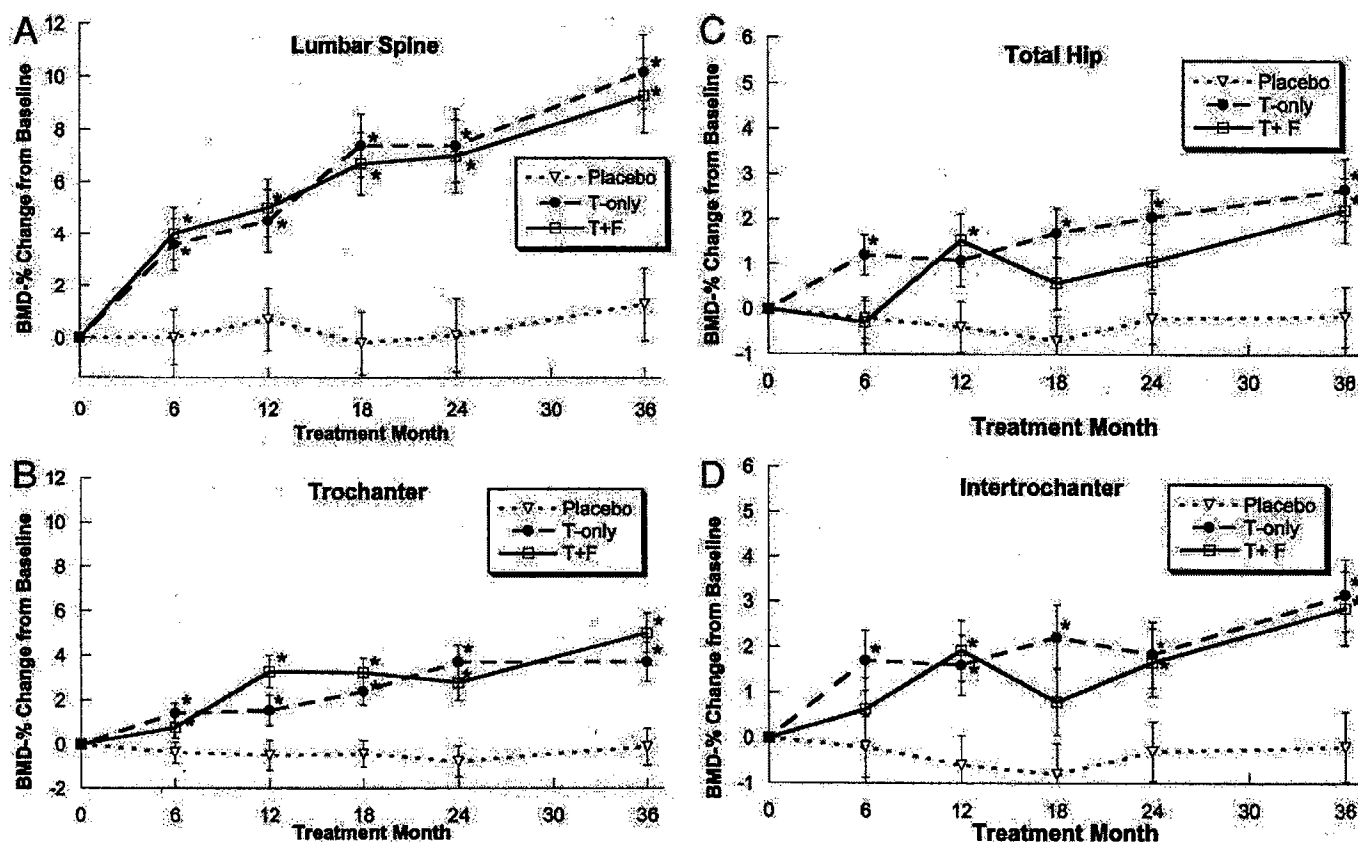
After 6 months of therapy, serum osteocalcin (a bone formation marker), intact PTH, and 25-hydroxyvitamin D did not change in any group (Table 3). In contrast, urinary deoxypyridinoline, a bone resorption marker, decreased significantly in both the T-only and T+F groups (both $P < 0.001$) but was unchanged in the placebo group ($P = 0.30$). Bone-specific alkaline phosphatase, a bone formation marker, decreased significantly in the T-only group ($P = 0.049$).

Prostate and hematological effects

Forty-nine of the 50 subjects who completed the 36-month study underwent end-of-treatment prostate ultrasound, and

TABLE 2. Mean BMD (g/cm^2) and 95% confidence interval measured by dual-energy x-ray absorptiometry in older men administered in T alone, T with F (T+F), or placebo at the lumbar spine, total hip, femoral neck, and intertrochanteric and trochanteric regions^a

| | Baseline | 6 months | 12 months | 18 months | 24 months | 36 months |
|---------------------------------|------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Lumbar spine | | | | | | |
| T only | 1.06 (0.98–1.13) | 1.09 (1.02–1.16) ^b | 1.10 (1.03–1.17) ^b | 1.13 (1.05–1.21) ^b | 1.13 (1.05–1.20) ^b | 1.16 (1.08–1.24) ^b |
| T+F | 1.03 (0.96–1.10) | 1.07 (0.99–1.14) ^b | 1.08 (1.00–1.15) ^b | 1.10 (1.02–1.17) ^b | 1.10 (1.03–1.17) ^b | 1.13 (1.05–1.20) ^b |
| Placebo | 1.04 (0.97–1.11) | 1.05 (0.97–1.12) | 1.05 (0.98–1.13) | 1.04 (0.97–1.12) | 1.05 (0.97–1.12) | 1.06 (0.98–1.14) |
| Total hip | | | | | | |
| T only | 0.96 (0.90–1.00) | 0.97 (0.91–1.02) | 0.96 (0.91–1.02) | 0.97 (0.92–1.03) ^b | 0.97 (0.92–1.03) ^b | 0.98 (0.92–1.04) ^b |
| T+F | 0.94 (0.89–1.00) | 0.94 (0.88–0.99) | 0.96 (0.90–1.01) | 0.95 (0.89–1.01) | 0.95 (0.90–1.01) | 0.96 (0.90–1.02) ^b |
| Placebo | 0.96 (0.90–1.01) | 0.95 (0.90–1.01) | 0.95 (0.90–1.00) | 0.95 (0.90–1.00) | 0.95 (0.91–1.01) | 0.95 (0.90–1.01) |
| Intertrochanteric region | | | | | | |
| T only | 1.11 (1.05–1.17) | 1.13 (1.06–1.19) | 1.13 (1.06–1.19) | 1.13 (1.07–1.20) ^b | 1.13 (1.07–1.20) | 1.15 (1.08–1.21) ^b |
| T+F | 1.11 (1.04–1.17) | 1.11 (1.04–1.18) | 1.13 (1.06–1.19) | 1.11 (1.05–1.18) | 1.12 (1.06–1.19) | 1.14 (1.07–1.21) ^b |
| Placebo | 1.11 (1.04–1.17) | 1.10 (1.04–1.17) | 1.10 (1.04–1.16) | 1.10 (1.03–1.16) | 1.10 (1.04–1.16) | 1.10 (1.04–1.17) |
| Trochanteric region | | | | | | |
| T only | 0.75 (0.70–0.79) | 0.76 (0.71–0.80) ^b | 0.76 (0.71–0.81) | 0.77 (0.72–0.81) ^b | 0.78 (0.73–0.82) ^b | 0.78 (0.73–0.83) ^b |
| T+F | 0.71 (0.66–0.76) | 0.71 (0.67–0.76) | 0.73 (0.68–0.78) ^b | 0.73 (0.68–0.78) ^b | 0.73 (0.68–0.78) ^b | 0.75 (0.69–0.80) ^b |
| Placebo | 0.74 (0.69–0.78) | 0.74 (0.69–0.78) | 0.74 (0.69–0.78) | 0.74 (0.69–0.78) | 0.73 (0.69–0.78) | 0.74 (0.69–0.79) |
| Femoral neck | | | | | | |
| T only | 0.78 (0.73–0.82) | 0.79 (0.74–0.83) | 0.78 (0.74–0.83) | 0.79 (0.74–0.84) ^b | 0.79 (0.74–0.83) | 0.79 (0.74–0.84) ^b |
| T+F | 0.74 (0.69–0.79) | 0.75 (0.70–0.79) | 0.76 (0.71–0.80) | 0.76 (0.71–0.81) | 0.76 (0.71–0.81) | 0.77 (0.72–0.82) ^b |
| Placebo | 0.81 (0.76–0.85) | 0.81 (0.76–0.85) | 0.80 (0.76–0.85) | 0.80 (0.76–0.85) | 0.81 (0.76–0.85) | 0.81 (0.76–0.86) |

^a Includes data from all measurements available at a given time point.^b $P < 0.01$ compared with baseline.**FIG. 2.** Mean percentage increase (\pm SEM) in BMD of the lumbar spine (A), trochanteric (B), total hip (C), and intertrochanteric (D) regions in older men with low T who were treated with either T (T-only), T and F (T+F), or placebo for 36 months. *, $P < 0.05$ compared with baseline and placebo.

all subjects had an end-of-treatment PSA. There was a small but significant increase in serum PSA in the T-only group ($P < 0.001$ by month 36), but there was no change in PSA in either the placebo or T+F group at any time during the study

(Table 4). Prostate volume increased significantly in all groups over the 3-yr study period. The increase in prostate volume in the T-only group was similar to the increase seen in the placebo treatment group ($P = 0.35$), whereas the in-

TABLE 3. Markers of bone metabolism during treatment (median, 75th–25th percentiles) in older men administered im T alone, T with F, or placebo after 6 months of therapy

| | Placebo | | T only | | T+F | |
|-----------------------------------|-------------|-------------|-------------|-------------------------|-------------|------------------------|
| | Baseline | Month 6 | Baseline | Month 6 | Baseline | Month 6 |
| Osteocalcin (ng/ml) | 2.06 (1.79) | 1.79 (2.44) | 2.35 (2.35) | 2.40 (1.37) | 2.38 (2.16) | 2.02 (2.08) |
| BSAP (IU/liter) | 14.3 (4.8) | 14.3 (5.2) | 14.6 (7.5) | 12.0 (5.2) ^a | 16.0 (5.6) | 13.9 (4.7) |
| U-deoxypyridinoline (μmol/mol Cr) | 5.8 (2.0) | 6.1 (2.5) | 6.2 (2.3) | 5.0 (1.6) ^b | 6.0 (2.0) | 4.5 (1.6) ^b |
| PTH (pg/ml) | 45 (28) | 42 (15) | 44 (28) | 50 (40) | 40 (19) | 52 (27) |
| 25 hydroxy-vitamin D (ng/ml) | 34 (20) | 44 (19) | 35 (21) | 39 (24) | 41 (12) | 48 (13) |

BSAP, Bone-specific alkaline phosphatase; U, urinary; PTH, intact PTH; Cr, creatinine.

^a $P < 0.05$ compared to baseline.^b $P < 0.001$ compared to baseline.

crease in prostate volume in the T+F group was significantly less than in the T-only group ($P = 0.02$).

One man in the placebo group, two in the T-only group, and no subjects in the T+F group had a diagnosis of prostate cancer, leading to discontinuation of study participation ($P = 0.46$). The two men with prostate cancer in the T-only group were diagnosed after 7 and 8 months of study participation. The indication for biopsy in one case was an abnormal digital rectal exam and in the other case was asthenia and fever. In both cases, one of six biopsy samples revealed Gleason grade 5 disease. For the man with the abnormal digital rectal exam, the positive biopsy occurred in the opposite lobe from the abnormal exam finding. The individual in the placebo group with prostate cancer was diagnosed after 24 months in the study; the indication for biopsy was an elevated PSA. Of the 17 remaining men who discontinued participation in the study, 15 were free of prostate cancer or other prostate diseases at the conclusion of the study. Two men were lost to follow-up.

Mean hematocrit and hemoglobin values increased significantly during treatment in the T-only and T+F groups ($P < 0.001$ compared with baseline and placebo) but were unchanged in the placebo group (Table 4). Increase in hematocrit was positively associated with elevations in T ($r = 0.41$; $P < 0.001$). In the T-only group, one man suffered a cerebral hemorrhage during treatment, and another man developed new symptoms of sleep apnea confirmed by a sleep study. There were no other serious adverse cardiovascular, cerebrovascular, or pulmonary events.

Discussion

This study demonstrates that im T therapy in older men with low serum total T levels increases BMD in the lumbar spine and hip over 3 yr. The increase in BMD would be expected to decrease fracture risk. The increases in BMD seen in this study are similar in magnitude both to those observed with T therapy in younger hypogonadal men (8–11) and to those seen with bisphosphonate therapy in men with osteoporosis (19, 20). The increases seen in BMD were not limited to the spine but also involved most areas of the hip that were measured. Previous studies of T therapy in older men have reported either smaller increases in lumbar spine BMD (12) or no increase in hip BMD with T therapy (12, 13). There could be a number of reasons why the findings of this study differ from the findings of these previous studies. For one, the men enrolled in this study all had baseline serum total T levels that were below the normal range for young men,

which was not the case in one of the previous studies (12). In addition, the serum levels of total T and E2 achieved with im T injections in this study were two to three times higher than those achieved in studies using T patches in older men (12, 13). Such large increases over baseline T and E2 levels might account for much of the difference between the increases in BMD seen in these studies. Although the dose-response range for bone in regard to T (or E2) in older men has not been established yet, these data may suggest that a certain threshold of serum T (or E2) must be reached and/or a certain magnitude of change from baseline levels must be achieved before significant effects of the sex steroids on bone are achieved in older men. It also is important to note that, in contrast to previous studies in older men, subjects in our study were not administered supplemental calcium and vitamin D. This may have increased the magnitude of the differences between placebo and treatment groups we observed in our study; however, dramatic improvements in BMD were seen without calcium and vitamin D supplementation. Whether supplementation in combination with T would result in even greater increases in BMD should be the subject of future research.

Both the T-only and the T+F groups had similar increases in serum nadir total T and E2 levels and in BMD; however, there was a significant decrease in serum DHT in the T+F group. This suggests that conversion to DHT is not essential for the effect of T on BMD. Because F incompletely blocks the conversion of T to DHT (21) and men in our study achieved at best a 50% reduction in serum DHT levels, it is still possible that low levels of DHT are required for stimulating increases in BMD.

The beneficial effects of T therapy on BMD may be mediated by its conversion to E2. The increases in E2 serum levels from baseline with T therapy in this study were substantial. The impact of E2 on BMD in men has been demonstrated in a man with aromatase deficiency who had high serum T levels but low BMD. Treatment with E2 resulted in epiphyseal closure and increased BMD (22). Furthermore, a second man with an E2 receptor mutation was found to have unfused epiphyses and low BMD (23). Other work has suggested that bioavailable E2 may be the best predictor of BMD in older men (24, 25). Although it is likely that E2 plays a major role in maintenance of BMD in men, further studies using nonaromatizable androgens will be required before we will completely understand relative roles of T and E2 in bone formation in men.

The mechanism by which androgens and/or estrogens

TABLE 4. Hematocrit, hemoglobin, prostate size, and PSA during treatment [means (95% confidence interval)]

| | Placebo | | | T-only | | | T+F | | |
|----------------------------------|------------------|------------------|-------------------------|------------------|-------------------------------|-------------------------------|------------------|-------------------------------|-------------------------------|
| | Baseline | Month 18 | Month 36 | Baseline | Month 18 | Month 36 | Baseline | Month 18 | Month 36 |
| Hematology | | | | | | | | | |
| Hematocrit (%) | 43.5 (42.3–44.8) | 42.7 (41.7–44.5) | 42.9 (41.5–44.4) | 42.5 (41.2–43.8) | 48.9 (47.4–50.4) ^a | 48.6 (47.1–50.0) ^a | 43.2 (41.8–44.5) | 48.2 (46.3–49.7) ^a | 47.4 (46.0–49.0) ^a |
| Hemoglobin (g/dl) | 14.7 (14.3–15.2) | 14.5 (14.2–15.2) | 14.6 (14.1–15.1) | 14.5 (14.1–14.9) | 16.5 (16.1–17.0) ^a | 16.6 (16.1–17.1) ^a | 14.6 (14.2–15.1) | 16.6 (16.1–17.0) ^a | 16.2 (15.7–16.7) ^a |
| Prostate | | | | | | | | | |
| Prostate size (cc ^b) | 32 (26–38) | ND | 42 (36–49) ^a | 29 (23–34) | ND | 43 (37–49) ^a | 33 (27–38) | ND | 38 (32–44) ^{a, b} |
| PSA (ng/ml) | 1.4 (1.1–1.9) | 1.5 (1.0–2.1) | 1.7 (1.2–2.3) | 1.0 (0.7–1.3) | 1.4 (1.0–2.0) ^a | 1.4 (1.0–2.0) ^a | 1.0 (0.7–1.3) | 0.8 (0.5–1.0) | 1.1 (0.8–1.6) |

ND, Not done. PSA is expressed as the geometric mean.

^a $P < 0.01$ compared with baseline.^b $P = 0.02$ compared with placebo and T-only.

improve BMD is unclear, but androgen receptors have been identified in osteoblasts (26). In our study, most markers of bone formation were unchanged, but the most sensitive marker of bone resorption (27), urinary deoxypyridinoline, decreased significantly. This suggests that T therapy reduces bone resorption more than it increases bone formation. This finding is in agreement with a recently published study in younger men (28). This “antiresorptive” effect of T also might be mediated by E2 (29) and is a main mechanism by which E2 is thought to increase BMD in postmenopausal women (30).

Regarding the prostate, all groups showed increases in prostate volume that were greater than those observed previously in prospective studies of older men (31, 32). The change in ultrasound equipment between baseline and the end of the study may have contributed to the seemingly larger-than-expected magnitude of volume change between baseline and end-of-study. However, because this would have affected equally all treatment groups, the relative volume change differences seen between treatment groups should still be valid. Notably, the T+F group had significantly less increase in prostate volume than either the T-only or placebo groups. The attenuation of prostate volume enlargement seen in this study with the concomitant use of a 5 α -reductase inhibitor, rather than a reduction in prostate volume that is usually reported with such therapy (32), is mirrored by the less-than-expected reduction in serum DHT levels in the T+F group and may have occurred because of the high serum T levels produced by the T-injection regimen used. Nonetheless, this attenuation of prostate growth by 5 α -reductase inhibition might be important in preventing symptomatic BPH and possibly reducing the risk of prostate cancer in older men treated with long-term T therapy; however, our trial lacked sufficient numbers of subjects to detect any possible benefit of F on the risk of these outcomes. Notably, the recently published prostate cancer prevention trial showed a 25% reduction in new cases of prostate cancer in older men treated with F therapy (33). Clearly, larger studies of T therapy with 5 α -reductase inhibitors in older men will be required before a small increased risk of prostatic complications can be excluded.

Subjects in our study did have a higher rate of erythrocytosis than seen in previous trials of T administration in older men using transcutaneous patches (10, 11). Thirty percent of subjects receiving 200 mg of TE every other week in our study developed a hematocrit greater than 52% and required a reduction in the T dose to an average of 158 mg. This finding is probably due to the high serum T levels, especially peak T levels, that were produced in this study and is consistent with rates of erythrocytosis seen in other studies in which older males have been treated with im T (34). Therefore, it is possible that a dose of 150 mg, rather than 200 mg, of TE every 2 wk might be a safer dosage in older men to prevent problematic erythrocytosis; however, there are not data to demonstrate that this dose will prevent bone loss. It is important to note that no ischemic strokes, heart attacks, or episodes of thromboembolism were observed in our study; however, this study lacked sufficient power to rule out a small increase in such events.

In summary, we conclude that T therapy in older men with

low serum T levels markedly increases BMD in both the spine and the hip over 3 yr. The addition of F to T does not diminish increases in BMD but does decrease prostate growth and increases in PSA compared with treatment with either T alone or placebo. Given its beneficial effects on BMD, larger, long-term randomized studies of T therapy with and without inhibitors of 5 α -reductase should be conducted to better define the risks and benefits of T therapy and its impact on the risk of osteoporotic fractures in older men.

Acknowledgments

We thank Arlen Sarkissian and Dorothy McGuinness for performing hormone assays and Robin Allen for clinical assistance.

Received June 30, 2003. Accepted October 20, 2003.

Address all correspondence and requests for reprints to: Dr. J. Lisa Tenover, Wesley Woods Health Center, 1841 Clifton Road Northeast, Atlanta, Georgia 30329-5102.

This work was supported by National Institutes of Health Grant AG10975.

References

- Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR 2001 Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. *J Clin Endocrinol Metab* 86:724–731
- Feldman HA, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviello AD, Bremner WJ, McKinlay JB 2002 Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts Male Aging Study. *J Clin Endocrinol Metab* 87:589–598
- Stanley HL, Schmitt BP, Poses RM, Deiss WP 1991 Does hypogonadism contribute to the occurrence of minimal trauma hip fracture in elderly men? *J Am Geriatr Soc* 39:766–771
- Jackson JA, Riggs MW, Spiekerman AM 1992 Testosterone deficiency as a risk factor for hip fractures in men: a case-control study. *Am J Med Sci* 304:4–8
- Abbasi AA, Rudman D, Wilson CR, Drinka PJ, Basu SN, Mattson DE, Richardson TJ 1995 Observations on nursing home residents with a history of hip fracture. *Am J Med Sci* 310:229–234
- Onghiphadhanakul B, Rajatanavin R, Chailurkit L, Piaseu N, Teerarungsikul K, Sirisriro R, Komindr S, Puavilai G 1995 Serum testosterone and its relation to bone mineral density and body composition in normal males. *Clin Endocrinol (Oxf)* 43:727–733
- Kenny AM, Prestwood KM, Marcello KM, Raisz LG 2000 Determinants of bone density in healthy older men with low testosterone levels. *J Gerontol A Biol Sci Med Sci* 55:M492–M497
- Katznelson L, Finkelstein JS, Schoenfeld DA, Rosenthal DI, Anderson EJ, Klibanski A 1996 Increase in bone mineral and lean body mass during testosterone administration in men with acquired hypogonadism. *J Clin Endocrinol Metab* 81:4358–4365
- Behre HM, Kliesch S, Leifke E, Link TM, Nieschlag E 1997 Long-term effect of testosterone therapy on bone mineral density in hypogonadal men. *J Clin Endocrinol Metab* 82:2386–2390
- Snyder PJ, Peachey H, Berlin JA, Hannoush P, Haddad G, Dlewati A, Santanna J, Loh L, Lenrow DA, Holmes JK, Kapoor SC, Atkinson LE, Strom BL 2000 Effects of testosterone replacement in hypogonadal men. *J Clin Endocrinol Metab* 85:2670–2677
- Wang C, Swerdloff RS, Iranmanesh A, Dobs S, Snyder PJ, Cunningham B, Matsumoto AM, Weber T, Berman N 2001 Effects of transdermal testosterone gel on bone turnover markers and bone mineral density in hypogonadal men. *Clin Endocrinol (Oxf)* 54:739–750
- Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Holmes JH, Dlewati A, Staley J, Santanna J, Kapoor SC, Attie MF, Haddad Jr JG, Strom BL 1999 Effect of testosterone treatment on bone mineral density in men over 65 years of age. *J Clin Endocrinol Metab* 84:1966–1972
- Kenny AM, Prestwood KM, Gruman CA, Marcello KM, Raisz LG 2001 Effects of transdermal testosterone on bone and muscle in older men with low bioavailable testosterone levels. *J Gerontol A Biol Sci Med Sci* 56:M266–M272
- Matzkin H, Chen J, Weisman Y, Goldray D, Pappas F, Jaccard N, Braf Z 1992 Prolonged treatment with finasteride (a 5 α -reductase inhibitor) does not affect bone density and metabolism. *Clin Endocrinol (Oxf)* 37:432–436
- Tollin SR, Rosen HN, Zurowski K, Saltzman B, Zeind AJ, Berg S, Greenspan SL 1996 Finasteride therapy does not alter bone turnover in men with benign prostatic hyperplasia—a Clinical Research Center study. *J Clin Endocrinol Metab* 81:1031–1034
- Matsumoto AM, Tenover L, McClung M, Mobley D, Geller J, Sullivan M, Grayhack J, Wessells H, Kadmon D, Flanagan M, Zhang GK, Schmidt J, Taylor AM, Lee M, Waldstreicher J, Pless Study Group 2002 The long-term effect of specific type II 5 α -reductase inhibition with finasteride on bone mineral density in men: results of a 4-year placebo controlled trial. *J Urol* 167:2105–2108
- Bosch JK, Hop WC, Niemer QH, Bangma CH, Kirkels WJ, Schroder FH 1994 Parameters of prostate volume and shape in a community based population of men 55 to 74 years old. *J Urol* 152:1501–1504
- Coakley FV, Hricak H 2000 Radiologic anatomy of the prostate gland: a clinical approach. *Radiol Clin North Am* 38:15–30
- Orwoll E, Ettinger M, Weiss S, Miller P, Kendler D, Graham J, Adams S, Weber K, Lorenc R, Pietschmann P, Vandormael K, Lombardi A 2000 Alendronate for the treatment of osteoporosis in men. *N Engl J Med* 343:604–610
- Ringe JD, Faber H, Dorst A 2001 Alendronate treatment of established primary osteoporosis in men: results of a 2-year prospective study. *J Clin Endocrinol Metab* 86:5252–5255
- Rittmaster RS 1997 5 α -Reductase inhibitors. *J Androl* 18:582–587
- Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. *N Engl J Med* 337:91–95
- Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med* 331:1056–1061
- Khosla S, Melton LJ, Atkinson EJ, O'Fallon WM, Klee GG, Riggs BL 1998 Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen. *J Clin Endocrinol Metab* 83:2266–2274
- Khosla S, Melton LJ, Atkinson EJ, O'Fallon WM 2001 Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men. *J Clin Endocrinol Metab* 86:3555–3561
- Colvard DS, Eriksen EF, Keeting PE, Wilson EM, Lubahn DB, French FS, Riggs BL, Spelsberg TC 1989 Identification of androgen receptors in normal human osteoblast-like cells. *Proc Natl Acad Sci USA* 86:854–857
- Ju HJ, Leung S, Brown B, Stringer MA, Leigh S, Scherrer C, Shepard K, Jenkins D, Knudsen J, Cannon R 1997 Comparison of analytical performance and biological variability of three bone resorption assays. *Clin Chem* 43:1570–1579
- Leder BS, LeBlanc KM, Schoenfeld DA, Eastell R, Finkelstein JS 2003 Differential effects of androgens and estrogens on bone turnover in normal men. *J Clin Endocrinol Metab* 88:204–210
- Falahati-Nini A, Riggs BL, Atkinson EJ, O'Fallon WM, Estell R, Khosla S 2000 Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men. *J Clin Invest* 106:1553–1560
- Barrett-Conner E 1997 Hormone replacement therapy. *BMJ* 317:457–461
- Williams AM, Simon I, Landis PK, Moser C, Christen-Barry W, Carter HB, Metter EJ, Partin AW 1999 Prostatic growth rate determined from MRI data: age-related longitudinal changes. *J Androl* 20:474–480
- McConnell JD, Bruskewitz R, Walsh P, Andriole G, Bieber M, Holtgrewe HL, Albertsen P, Roehrborn CG, Nickel JC, Wang DZ, Taylor AM, Waldstreicher J 1998 The effect of finasteride on the risk of acute urinary retention and the need for surgical treatment among men with benign prostatic hyperplasia. Finasteride Long-term Efficacy and Safety Study Group. *N Engl J Med* 338:557–563
- Thompson IM, Goodman PF, Tangen CM, Lucia MS, Miller GJ, Ford LG, Liever MM, Cespedes RD, Atkins JN, Lippman SM, Carlin SM, Ryan A, Szczepanek CM, Crowley JJ, Coltman Jr CA 2003 The influence of finasteride on the development of prostate cancer. *N Engl J Med* 349:215–224
- Hajjar RR, Kaiser FE, Morley JE 1997 Outcomes of long-term testosterone replacement in older hypogonadal males: a retrospective analysis. *J Clin Endocrinol Metab* 82:3793–3796

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.



WORLD ANTI-DOPING AGENCY

INDEPENDENT OBSERVER REPORT

TOUR DE FRANCE 2003

TABLE OF CONTENTS

| | |
|---|-----------|
| 1. INTRODUCTION | 4 |
| 2. INITIAL PREPARATIONS AND MEETINGS | 4 |
| 3. MEDICAL CHECK-UP | 6 |
| A. BLOOD TESTS | 6 |
| B. MEDICAL EXAMINATION | 9 |
| 4. OUT-OF-COMPETITION TESTING BEFORE THE START | 10 |
| 5. IN-COMPETITION TESTING | 11 |
| A. SELECTION PROCESS | 13 |
| B. NOTIFICATION | 13 |
| C. ESCORTS | 14 |
| D. TESTING AREA | 14 |
| E. PROCEDURES FOR TAKING SAMPLES | 14 |
| F. TRANSPORT | 16 |
| 6. OUT-OF-COMPETITION TESTING DURING THE TOUR | 19 |
| 7. LABORATORY | 20 |
| 8. HANDLING THE RESULTS | 27 |
| A. WHILE THE OBSERVERS WERE PRESENT | 27 |
| B. AFTER THE OBSERVERS' DEPARTURE | 27 |
| 9. INFORMING AND EDUCATING ATHLETES | 30 |
| 10. UCI MEDICAL COMMISSION | 31 |
| 11. MISCELLANEOUS | 32 |
| 12. CONCLUSIONS | 32 |
| 13. ACKNOWLEDGEMENTS | 34 |

| | |
|--|-----------|
| 14. MEMBERS OF THE INDEPENDENT OBSERVERS TEAM | 34 |
| 15. APPENDICES | 36 |
| APPENDIX 1: PROCEDURE FOR DETERMINING BLOOD PARAMETERS (UCI) | 36 |
| APPENDIX 2: MEASUREMENT PROTOCOL (UCI) | 38 |
| APPENDIX 3: INFORMATION FOR RIDERS (UCI) | 39 |
| APPENDIX 4: REQUEST FOR INFORMATION 04/08/03 | 40 |
| APPENDIX 5: UCI REPLY (07/08/03)..... | 41 |
| APPENDIX 6: UCI REPLY (06/08/03)..... | 42 |
| APPENDIX 7: FAX TO THE CPLD (18/08/03)..... | 43 |
| APPENDIX 8: REPLY FROM THE CPLD (18/08/03)..... | 44 |
| APPENDIX 9: ASO INFORMATION LEAFLET FOR RIDERS | 45 |
| APPENDIX 10: TOUR DE FRANCE CODE OF ETHICS | 47 |

1. INTRODUCTION

This report is submitted by the team of Independent Observers (IO) from the World Anti-Doping Agency (WADA), which was present at the Tour de France 2003. The IO team was able to observe the anti-doping programme implemented by the various organisations working in cooperation during the Tour: The International Cycling Union (UCI), the French Ministry of Sport, the Amaury Sport Organisation (ASO), the French Council for the Prevention and Fight against Doping (CPLD) and the French National Drug Testing Laboratory (LNDD).

The team appointed by WADA to carry out this task comprised 3 members, all regarded as experts in their particular fields.

2. INITIAL PREPARATIONS AND MEETINGS

In the run up to the 2003 Tour, WADA circulated an agreement among the relevant parties in order to confirm that, in accordance with the mandate of the IO programme, the observers would have access to all the relevant documentation and would be able to observe the anti-doping control process implemented for the Tour de France at its various levels. In spite of a few initial problems associated with the legal constraints specific to the Code of Public Health in France hosting the Tour, it was possible to reach an agreement authorizing the WADA team to observe the following procedures:

- selection procedures;
- notification of the cyclists selected for the controls;
- analysis of samples at the LNDD;
- preparation of the controls;
- compiling of the appropriate forms after the controls;
- preparing the sample for dispatch to the laboratory;
- procedures in the event of a "B" sample.

In addition to this, the observers were able to enter the doping control station, provided that they were medical doctors and there was adequate room to accommodate the cyclist, the doctor taking the sample, the UCI delegate and possibly the accompanying person of the cyclist.

An anonymous copy of the doping control forms, as well as a copy of the analysis reports were also supplied to the observers.

Before the observers arrived in Paris, arrangements were made for several meetings to be held in order to ensure, on the one hand, that just as events were starting off, all the parties concerned would be informed of each other's role in the process, while also ensuring that there would be no problems from an organisational point of view.

Two important meetings were held during the first two days, on 1st and 2nd July, in Paris. During the first meeting between a representative from WADA and M. D. Baal, Deputy Director of the Tour, the entire logistics for the event, including accommodation, transportation, etc. were reviewed and coordinated. A car and driver were made available to the observers from the time they arrived in Paris.

A second meeting took place between representatives from the French Ministry of Sport, CPLD, UCI, ASO, LNDD and the WADA observers, which focused on this first contact being made and on explaining the WADA observers' mission, their objectives and role during a sporting event. All the steps involved in the anti-doping test process implemented during the Tour were described in detail and explained. Furthermore, an agreement was reached on guaranteeing anonymity with regard to the copies of reports sent to the observers.

It is important to note that during the meeting the CPLD expressed its opinion about the exchange of information with the observers relating to the doping control forms. It did not agree with the compromise offered by the French Ministry of Sport and consequently, the CPLD stated that it declined all responsibility in the event of any dispute.

During the meeting the President of the IO team emphasised that this type of mission is carried out based on a positive, constructive approach. These objectives can only be achieved with the cooperation of all the present parties based on a system of ongoing communication to help avoid any problems during the mission.

During this meeting, the President also gave all the relevant authorities copies of the documents guaranteeing confidentiality with regard to the information gathered as part of the mission and to the commitment to expose any personal conflict of interest.

On the evening of 4th July, the WADA observers were invited to attend the general reception for the teams being held at Paris City Hall. Race officials not only made the most of this occasion to inform the teams about the Tour de France's Code of Ethics, along with its regulations on behaviour and safety, but they also made the cyclists and their backup teams aware of the issue of doping¹. The anti-doping testing process was quickly explained and an appeal was made to the cyclists for maintaining the spirit of fair play.

RECOMMENDATIONS

It is essential that in future, agreements between the various parties are reached and signed well beforehand in order to guarantee the best possible organisation for all the parties concerned. The agreement was sent to the relevant parties on 16th May 2003 and it was only on 27th June, virtually just before the observers were due to arrive that agreement was successfully reached.

3. MEDICAL CHECK-UP

Before the start, all the cyclists underwent a medical check-up, including a blood test and a medical examination.

a. BLOOD TESTS

All 198 riders officially registered for the 2003 Tour de France underwent a blood test the day before the start of the race.

The samples were taken at the hotels where the teams were staying from 7.30 AM onwards. These tests were carried out under the responsibility of the UCI, which appointed several teams made up of a doctor to take the sample and two UCI commissioners.

¹ See pages 30-31 of this report for more details

Three samples were taken from each rider: an A sample and a B sample for evaluating the levels of haematocrit, haemoglobin and the percentage of reticulocytes and free plasma haemoglobin; a third sample for evaluating other biological parameters (transaminases, glucose, iron, transferrin, ferritin, cortisol, etc.).

The IO team members observed the procedures for taking the blood samples, compiling the forms and transporting the samples to a temporary laboratory located in a hotel. There were two different teams working in the laboratory. One was from the Lausanne Laboratory and the other from the Ghent Laboratory. Each team was made up of a Scientific Director and a Technician. The Swiss team used a Sysmex ® analyzer, while the Belgian team used a Coulter ACT 8 ®. The laboratory was also equipped with a centrifuge and a Hemocue® analyzer for measuring free plasma haemoglobin. This is the first occasion where the UCI has not only evaluated the usual parameters (haematocrit, haemoglobin and the percentage of reticulocytes), but also free plasma haemoglobin, which rises quite significantly when synthetic haemoglobin is administered.

When the analyses, which were carried out immediately as soon as the samples arrived, showed abnormal profiles (abnormal values or trends), the UCI questioned the rider (or his doctor) about the source of this abnormality, or let him know that he would have another test carried out during the race and that he would be classified as being suspect by the UCI Anti-doping Commission.

The UCI authorised the observers to attend all the health check procedures, but not when the results were being issued. The reason behind that being that the health checks were not part of the anti-doping process. Nevertheless, the observers do not share this view because on several occasions during the Tour, the UCI carried out tests in competition and out of competition based on suspect results from blood tests taken as part of the health checks.

This strategy paid off with a out-of-competition control during the Tour, which showed a positive result for erythropoietin (EPO).

Since this year, the UCI has been using a new protocol for identifying riders with an abnormal blood profile. If, for instance, a rider has a haematocrit value of 48% (below 50%), but if the average value of the four previous samples taken was 43%, the Anti-doping Commission will automatically make this rider provide a urine sample to be screened for EPO.

The two doctors included in the IO team were able to view the UCI's blood parameters database and listen to the explanations from Mario Zorzoli, the UCI doctor, about the strategy linked to the health checks.

Appendices I and II describe the procedure for the medical examination carried out to determine blood parameters and the protocol for measuring free plasma haemoglobin. Appendix III contains the letter sent to all the riders before the race containing some information about the blood tests.

The third blood sample was dispatched in refrigerated form to a laboratory in Switzerland for an evaluation of the other parameters, which would be passed on to the UCI doctors at a later stage.

All the technical and administrative procedures were carried out quickly, in a highly professional manner and with the excellent cooperation of all the riders.

During the health checks the commissioners requested health booklets from all the riders for them to photocopy. During the technical meeting on 4th July the UCI Medical Commission returned the health booklets and informed the team representatives of the results from the health checks.

One member of the IO team was also present at the health checks carried out on 9th July, where all the riders in the six teams were examined.

During the Tour the UCI carried out other health checks after the observers' departure, where all the riders with suspect results had to undergo a out-of-competition control or a control on the same day at the end of the stage in order to screen for erythropoietin.

The day before the first health check, the UCI held a meeting attended by the doctors involved in taking the samples and the laboratory managers to explain to them their duties and responsibilities and to issue the relevant documentation and equipment to them.

The observers would like to congratulate the UCI for implementing this strategy involving health checks, which is still at the moment and was in the past a very important measure for protecting the riders' health. The introduction of testing for abnormal biological profiles and the evaluation of free plasma haemoglobin demonstrate that the UCI strives to improve its health check procedures.

Based on the analysis of the individual assessments, it is possible to divide the riders into three categories:

- Those who will be banned from starting due to a haematocrit level above 50% and a haemoglobin level higher than 17 g/dl;
- Those with no biological abnormalities;
- Those who will be authorised to start but, due to a biological profile regarded as "suspect", will have to be included in the group of riders obliged to take a urine test to be screened for EPO at the end of the prologue.

RECOMMENDATIONS

- *During any future WADA observation mission, the UCI should supply the results from the health checks to the WADA team to prove the system's effectiveness (total, indisputable transparency).*
- *The UCI should provide a copy of the completed form with the sample codes for each rider.*
- *Samples should be transported in refrigerated form in a sealed case, along with an appropriately completed security document.*

b. MEDICAL EXAMINATION

On 3rd and 4th July all the riders underwent a medical examination organised by the medical team employed by the ASO led by Dr. Gérard Porte.

The medical team's aim was to make an initial contact with all the riders. It comprised six doctors and two nurses.

A medical record was completed for each cyclist, who underwent several observations and examinations: weight, height, spirometry, cardiopulmonary auscultation, blood pressure and ECG.

The technical procedure used for measuring weight and height was not appropriate, nor were the conditions ideal for cardiopulmonary auscultation (the rooms in the large hall of the Palais des Expositions did not have integrated ceilings and there was background noise. The cars following the Tour were also located nearby). The cyclists' privacy was not respected either. For instance, the ECGs were carried out in a large room with four beds and no curtains separating them from the ever-present media.

It is vital and necessary for contact to be made between the medical team in charge during the Tour de France and the cyclists and their doctors, but some basic principles of medical practice need to be observed.

RECOMMENDATIONS

- *The cyclists' privacy should be respected.*
- *Examinations and, in particular, recording the cyclists' medical history should be carried out in an atmosphere of peace and quiet in order to obtain as much information as possible from the cyclists.*
- *The procedures for measuring weight and height should comply with normal technical procedures.*
- *It is vital that the cyclist's medical record is supplied and that the team doctor is interviewed.*
- *Closer cooperation with the UCI medical team and the issuing the results of the blood analyses carried out in Switzerland will definitely benefit this large medical structure put in place for the Tour de France.*

4. OUT-OF-COMPETITION TESTING BEFORE THE START

The day before the start, the UCI decided to control at random two cyclists who had "suspect" biological profiles.

On the morning of the prologue another cyclist was also tested. The three riders' urine samples were screened for erythropoietin.

The IO team was present at one of the tests carried out by a team made up of the Anti-doping Inspector and a doctor from the UCI. The test was carried out in accordance with the technical and administrative procedures set out by the UCI's Anti-doping regulations. The three samples were transported to the laboratory along with the samples taken after the prologue, which was 34 hours after the first out-of-competition sample was taken.

The observers did not see the conditions for storing and ensuring the security of these three samples taken during a random test between the time they were taken and their arrival at the venue for the anti-doping test during the prologue.

The observers agree with the UCI's strategy of testing at random cyclists with "suspect" results from the health checks.

RECOMMENDATIONS

The observers recommend that the samples are transported immediately to the laboratory after being taken and not 34 hours afterwards.

5. IN-COMPETITION TESTING

The French Ministry of Sport, the UCI and ASO shared responsibility for in-competition testing. The Ministry of Sport appointed a doctor responsible for all the samples taken.

The selection of the athletes took place every day one hour before the finish in the UCI Anti-doping Inspector's car in the presence and with the cooperation of the doctor appointed by the Ministry of Sport.

In accordance with UCI Anti-doping regulations, at each stage, the wearer of the yellow jersey and the stage winner were automatically selected to take an anti-doping test. During several stages the Anti-doping Inspector received a message from the President of the UCI Anti-doping Commission instructing him to select directly a few riders with "suspect" profiles from the blood tests.

Usually three other riders were then selected randomly to give a total of six or seven riders to be tested and two reserve riders. Half an hour before the finish, the anti-doping inspector would give the President of the board of commissioners the jersey numbers of the riders selected for testing, as well as those of the reserve riders. This information would then be passed on by the President via the Tour radio (accessible to Tour officials) twenty minutes before the finish. The names and numbers of the riders were then displayed at the entrance to the anti-doping control station.

During the time-trial stages lots would be drawn before the first rider set off and notice would be given by a UCI commissioner five minutes before each selected rider set off. After the finish the rider had an hour to report to the doping control station without ever being accompanied.

The Ministry of Sport doctor took the urine samples in two caravans made available by the ASO, one of which was used as a waiting room. The doping control station was at least 50 metres from the finish line and the press rooms. It was surrounded by barriers with a door opening onto the course. There was always a security guard from the organisation at the door.

The Berlinger ® system was used to take the samples and the forms from the Ministry of Sport were used as report templates.

Once all the tests were carried out, the doctor taking the samples and the Medical Inspector would put all the in-competition samples and the out-of-competition samples from the same day in a case containing dry ice required for transporting the samples at minus 20 degrees Centigrade.

The case was immediately brought to the heliport or airport where a helicopter or plane chartered by the organisation was waiting each evening to take the case and the samples to Bourget airport in Paris where they were handled by a private carrier. This carrier brought the samples between 0900 and 1000 the following day to the Châtenay-Malabry anti-doping laboratory.

The IO team observed the system for selecting the riders, the notification system, the procedures for taking the samples and the team also monitored the riders after the finish until they reported to the testing area during the prologue and the first four stages. The observers also monitored the case containing the samples from the doping control station right to the premises of the company assigned with their transportation during the day of the prologue. At the end of anti-doping testing after the third stage, the observers monitored how the case was brought to the heliport.

During the Tour, 132 urine samples were taken in-competition (6 samples for fifteen stages and 7 samples for 6 stages).

Most of the procedures followed during the in-competition tests complied with the UCI Anti-doping regulations and/or WADA International Standard for Testing. However, the observers identified a few discrepancies with regard either to the UCI Anti-doping regulations or the International Standard for Testing.

a. SELECTION PROCESS

When the riders were selected during the second stage, one of those selected by the President of the UCI Anti-doping Commission was not notified at the finish due to an error when noting the numbers of the riders selected. During the selection process at the third stage, the inspector had made a mistake noting the figures in the jersey number of the selected rider. Because of the confusion from the previous day when a rider was omitted, the latter was selected automatically for testing that day by the President of the UCI Anti-doping Commission.

b. NOTIFICATION

During the prologue the cyclists were notified five minutes before the start. This meant that the cyclists who were not notified then still had the opportunity to take a stimulant before the start of the race, as they were certain not to be tested (unless they won!). Of the six cyclists tested during the prologue only one reported for testing more than 60 minutes after notification, without any comment from the inspector. The Anti-doping Inspector informed some of the athletes and managers that they had 60 minutes to report for testing.

During the road-racing stages notification was given via the Tour-radio 20 minutes before the end of the race. This meant that the riders who were not selected again had an opportunity to take a fast-acting stimulant because they knew for certain they would not be tested (unless they won!).

c. ESCORTS

There were no escorts. The cyclists sometimes took over 20 minutes to get changed in their team trucks. Some kind of manipulation could have taken place.

d. TESTING AREA

There was no sign available to indicate where the anti-doping control station was. In addition, the testing area's location was not indicated in the route guide. The anti-doping caravan (waiting room and area where samples were taken) and the relevant doctor's car had a "Contrôle Médical" (Medical Test) sign rather than any mention of anti-doping.

In one instance, a cyclist and his doctor were meant to report for the anti-doping test, but could not find the venue due to the lack of signs.

The premises where the tests were carried out were far too small. There was also no system for recording when people came and left the area.

Every day there were unauthorised people in the anti-doping control and waiting areas, such as chauffeurs, mobile-home drivers and sometimes even members of the media.

With a UCI doctor's authorisation, a television crew was able to enter the restricted area and film the doping control station with only a WADA observer present. Afterwards, the TV crew remained in the restricted area around the caravan and filmed cyclists leaving the testing area, as well as the inside of the testing and waiting areas when a cyclist was there. Finally, the TV crew filmed a close-up of the procedure for filling and closing the dry ice container.

e. PROCEDURES FOR TAKING SAMPLES

Cyclists did not receive at any time an explanation of what the anti-doping test procedures involved.

Often the doctor taking the sample carried out himself the process for filling the samples without requesting the cyclist's authorisation or recording this action on the test form. Given the numerous (7) copies of the report which had to be made, the last copy, intended for the laboratory, was on several occasions virtually illegible.

The cyclist's privacy was not respected during micturition. This was carried out with the Anti-doping Inspector present, while the small bathroom was used to store the equipment and samples after they had been taken.

On at least one occasion, the doctor taking the sample left the caravan when the cyclist was trying to urinate. The Anti-doping Inspector was not able to observe the cyclist from where he was, although this was not part of his function.

The doctor taking the samples never measured their density and pH.

According to the doctor taking the samples, pH and density are not measured because of a directive from the LNDD (French National Drug Testing Laboratory) issued two years ago stating that this measure was not necessary.

The Independent Observers, however, questioned the Ministry of Sport on this matter, which informed them that neither UCI regulations nor French law made this measure compulsory, even if there was a relevant box for this purpose on the form and that it was stipulated by the Olympic Movement Anti-doping Code.

Whenever there was a sample containing an insufficient quantity of urine the doctor taking the samples never used the Berlinger® system intended for this purpose (with blue caps). A cyclist held his open sample in his hand, at one moment he was even left all alone in the doping control station. Despite this, the doping control form provides a section for indicating the number of the intermediate seals, which was therefore never used.

The doctor taking the samples always observed the 75 ml limit. Sometimes, when analysing EPO, a larger quantity of urine is required. To obtain this it would have been preferable to pour more urine into the bottles even if there was already a sufficient quantity of urine.

On several occasions the doctor took more than 75 ml urine, the quantity taken was noted in the report. He then filled bottle A and bottle B up to the label and the rest of the urine was poured down the toilet.

The doctor taking the samples noted all the drugs featuring in the rider's health booklet on the form and asked him to state all the drugs he had recently taken. He did not ask him to state the nutritional supplements he had taken. The doctor often did not ask the rider if he had any comments to make about the procedure.

After a cyclist was tested the samples were not kept in a safe, refrigerated place. The Berlinger® set with the sample was placed on the ground in the small bathroom in the caravan, which was not used for micturition.

During the prologue controls, the three samples from the random control taken in the morning were kept refrigerated in the carry case, which was not sealed and was located outside the caravan near the entrance.

In one case, the cyclist's copy of the doping control form was detached before the Cycling Director could sign the form. He signed it afterwards but the cyclist's copy did not have his signature on it. The various copies were put in envelopes and sent by normal post to the relevant authorities. The copies were therefore not kept in sealed envelopes.

Once the in-competition tests were complete, the container of dry ice was emptied in the street in full public view, with the samples placed on the ground nearby.

Then all the samples were placed in the container again and the driver of the doctors' car put the dry ice back in the container using plastic bags to protect his hands.

The actual container was not sealed. The unsealed envelope containing the copies of the laboratory reports and the sample security form were placed under the lid of the container, which did not close properly. This envelope was therefore in full public view and easily accessible.

f. TRANSPORT

There was no transport form (security form for the case).

After the prologue, the doctor taking the samples and the Anti-doping Inspector took the container to their hotel. Immediately when they arrived, the container was given to an employee from Dynaposte® (who was not requested for proof of identity). This company did not supply the doctor taking the samples with a transport form. This meant that the doctor had no proof that the samples had actually been delivered. Dynaposte® transported the samples to a post office where they were kept in air-conditioned premises. The post office's security system consisted of an alarm and access code. Dynaposte® does not have a quality control system. The samples were scheduled to be dispatched to the laboratory at 9 AM the following morning.

RECOMMENDATIONS

The following measures are recommended by the IO team as a means of improving the anti-doping test procedures at the Tour de France:

- The procedures for selecting the riders should be carried out in an atmosphere of peace and quiet to prevent any mistakes being made.*
- UCI Anti-doping regulations should describe precisely when the riders should be notified during the time-trial and road-racing stages. The form described in the UCI Anti-doping regulations should only be used for notification after the finish, preferably in the mixed area or alternatively, beside the relevant team trucks.
The Observers believe that this system can be totally practicable, even during a major competition like the Tour de France.*
- Once notification has been given, an escort trained specially for this purpose should accompany the rider until he arrives at the anti-doping control station, as described in the UCI Anti-doping regulations (Article 53) and in accordance with Article 5.4 of the International Standard for Testing.*
- The time the rider has to report for testing specified in the UCI Anti-doping regulations (Article 54 – 30 minutes or 50 minutes, if he has to attend a press conference) should be observed.*
- The testing area should be clearly signposted from the finish line (Article 38 of the UCI Anti-doping regulations).*

- *The testing area should comply with UCI recommendations (Article 39), especially with regard to its dimensions, guaranteeing riders' privacy during the test, as well as with Article 6.3 of the International Standard for Testing.*
- *The person appointed to guard the entrance to control station should have a system for recording who enters and leaves the area. The press, organisation drivers, mobile home drivers and other persons not involved in the anti-doping test process should not enter the testing area (Article 40 of the UCI Anti-doping regulations).*
- *The doctor taking the samples should explain the procedures to the riders, give them the opportunity to ask questions and take all the samples in a calm environment and in accordance with Article 47 of the UCI Anti-doping regulations and the procedures specified in WADA's International Standard for Testing (Article 7.0).*
- *The doctor taking the samples must ask riders which nutritional supplements they have taken, as this information could help with the interpretation of a positive analysis report and the decision on what kind of sanction to impose (Article 10.5 of WADA's World Anti-doping Code).*
- *The french doping control form should be amended so that there are fewer copies or that the copy to be sent to the Laboratory is the third or fourth sheet so that it is more legible. The IO team does not understand why one copy has to be supplied to the National Federation and a second to the International Federation.*
If it is an international competition the copy should be given to the International Federation and if it is national it should be given to the National Federation. The IO team wonders why a copy has to be supplied to the French Ministry of Sport if the French Council for the Prevention and Fight against Doping already receives one.
- *The doctor taking the samples should pour all the urine collected into bottles A and B because it is sometimes the case that the laboratory needs a large amount of urine to be able to confirm a quantifiable substance or detect erythropoietin.*
- *The samples should be kept refrigerated in a secure place after they have been taken.*

- *The doctor taking the samples and the UCI Anti-doping Inspector should place the copies of the report in the envelopes intended for the various recipients after the last sample has been taken and close them using the security seals (Article 66 of the UCI anti-doping regulations).*
- *The doctor taking the samples should draft a report after each doping control session. A single report at the end of the Tour mission would not allow for corrective measures to be taken if there were irregularities in one of the controls during the actual Tour.*
- *The samples and envelope containing the reports to be sent to the laboratory should be placed in a case, which should be sealed by the doctor taking the samples and the Anti-doping Inspector.*
- *The doctor taking the samples or the Anti-doping Inspector should complete a security document for the case (in accordance with Article 9.3.2. of the International Standard for Testing), specifying the date and time at which the case was sealed and the security seal number. The integrity of each person who will carry the case should be guaranteed and a new entry must be made in the security document when the case is received.*
- *The Tour's organisation should choose a company with a quality assurance system to transport the case.*
- *The samples should be transported in a case refrigerated at a temperature between 0°C and 10°C while in transit.*
Transporting the samples at -20°C is secure but it slows down the procedures for preparing the samples in the laboratory as they have to wait until the samples have thawed once they have arrived.

6. OUT-OF-COMPETITION TESTING DURING THE TOUR

A total of seven out-of-competition controls were carried out during the Tour de France (one on 7th July, two on the first rest day, one on 18th July and three on the second rest day). The doctor from the Ministry of Sport taking the samples carried out the controls in cooperation with the UCI Medical Inspector.

The President of the UCI Anti-doping Commission decided on the riders to be selected and the laboratory screened all the samples taken for erythropoietin.

One of the WADA observers observed one of the controls carried out at 7 AM on 7th July. It was carried out at the rider's hotel. The only remarks to be made relate on one hand to the time the rider took to report for the control – 23 minutes – after being notified by one of the team managers without being accompanied by the UCI Medical Inspector and on the other hand to the system for transporting the samples. After taking the samples, the doctor carried them in a small bag.

RECOMMENDATIONS

- *The UCI and French Ministry of Sport should carry out more controls, especially to monitor riders with "suspect" blood profiles, instead of monitoring them by selecting these riders for in-competition controls. Post-competition proteinuria can make it more difficult to interpret the results from the procedure for detecting erythropoietin in the laboratory.*
- *The UCI Medical Inspector should accompany the team manager from the time of their meeting until the rider is notified.*
- *The samples should be transported and stored in a refrigerated case, closed with a security seal until the time it is finally transported to the laboratory.*

7. LABORATORY

The analyses of all the samples taken for anti-doping testing, in competition and out of competition during the Tour de France, were carried out by the French National Drug Testing Laboratory (LNDD) in Châtenay-Malabry.

The LNDD is a national public, administrative institute, which operates under the responsibility of the Ministry of Sport.

This laboratory carries out analyses under the terms of Article L.3632-2 of the French Public Health Code and is responsible for managing and sending the equipment required to take the samples, as specified in the article of the Decree of 11th January 2001 mentioned above. Another of the LNDD's tasks is to carry out research in the area of doping prevention.

The laboratory has evolved historically as part of the institutional framework represented by national and international sporting bodies (sports federations, International Olympic Committee and World Anti-Doping Agency) and administrative bodies in the form of the Ministry of Sport and more recently (1999) the Council for the Prevention and Fight against Doping (CLPD).

In view of this, the laboratory's activities meet the requirements of national regulations and those of the sporting bodies.

At the moment, the LNDD is the only establishment in France approved by the IOC and WADA for carrying out analyses of anti-doping controls. In order to maintain the quality level of the service provided, the remuneration of the staff involved in carrying out the analyses does not depend on the number of samples processed nor on the results of these analyses. The LNDD is situated in the Châtenay-Malabry centre for popular education and sport (CREPS). It is made up of three technical departments, a paratechnical department, a quality assurance department, as well as a general secretariat. It has a current capacity for processing around 9,000 samples annually, based on 800 (between 700 and 900) per month over 11 months, taking into account a period of one month to deliver the results from the time the samples are received at the laboratory.

The Laboratory's management team is made up of the Director of the Laboratory, Prof. Jacques de Ceaurriz and a General Secretary. The Director is the laboratory's technical manager.

The LNDD has a staff of 40 comprising:

- 1 Director
- 1 General Secretary
- 3 Heads of department
- 1 Quality Assurance manager
- 23 technicians and 3 para-technical staff

- 8 administrators.

The laboratory has three technical departments. The various heads of department are also the technical managers for their respective departments:

- Department of GC chemical testing. This testing department is responsible for carrying out conventional analyses using gas chromatography, with or without mass spectrometry.
- Department for analytical research and development and LC chemical testing. Its function is to develop new analytical methods for identifying new doping products, as well as to improve already existing ones.
- Department for biological research and development and immunochemical testing. Its function is to develop new biological analytical methods for identifying new doping products, as well as to improve already existing ones. This department is responsible for the technical procedures of erythropoietin screening.

The laboratory's quality control system has had general accreditation from COFRAC (French Committee for Accreditation) in Medical Biology (no. 1-1174) since 1st June 2001.

The LNDD currently has the following equipment:

11 units – GC/MS

3 units – GC/MS-MS

2 units – (LC/MS)

2 units – system for analysing luminescence with dark room and optical system

1 unit – (LC/MS-MS)

An observer from the IO team visited the LNDD on the morning of 10th July. The observer was very warmly welcomed by the Director and quality assurance manager. He was able to visit the laboratory facilities, which are currently under renovation. These facilities are quite extensive offering an ideal separation between all the departments and sections. For instance, each technical department has a screening section, confirmation section and research section.

The observer witnessed the reception of the samples taken during the 4th stage, which arrived at the laboratory via Dynaposte® at around 10 AM.

The procedure for receiving the samples was carried out in a highly professional manner, in accordance with the laboratory's quality system and WADA's International Laboratory Standards. The samples were frozen at the time of their reception.

The observer was able to look at some of the laboratory's quality documents. The quality control system is well structured and implemented in a very active and highly professional manner.

The IO team concluded that the analysis reports described the methods used for screening and confirmation, but that these reports did not mention the technical procedure codes used.

The IO team also concluded that there were a few weaknesses concerning the security system. For example, a door leading outside remained open while the samples were being received; the WADA observer was not asked for identification at the entrance to the laboratory and his presence was not recorded.

The laboratory sign at the entrance on avenue Roger Salerno was not very visible, which made it difficult for any visitor trying to locate it.

During the Tour de France the Laboratory made small changes to the way in which it organised its daily activities. The samples from the Tour de France were processed as a priority and working hours were extended slightly, but the laboratory was not open at night or during the weekend.

Outside normal working hours and during the weekend, there was nobody at the laboratory, not even a security guard. The laboratory does, however, have a double security system:

- centrally controlled anti-burglary shutters on all the windows
- an alarm linked to a remote monitoring and response centre (at the Director or General Secretary's request).

While the WADA IO team was present at the Tour, following a telephone call between 3 and 4 PM, the laboratory sent the analysis reports by fax, ensuring anonymity was preserved, to the President of the IO team.

After the IO team's departure the Laboratory sent the analysis reports every day via the IO team President's confidential fax number in Lisbon.

The analysis reports were sent to the UCI Anti-doping Commission and the President of the French Council for the Prevention and Fight against Doping (CPLD) and for information, to the President of the International Olympic Committee's Medical Commission and to the person dealing with these matters at the French Cycling Federation.

During the Tour de France the LNDD processed a total of 142 samples (132 taken in-competition and 10 out-of-competition). In 2002, 138 samples were taken and 170 samples in 2001.

The in-competition samples taken during the prologue, 2nd, 4th, 6th, 7th, 8th, 9th, 12th, 13th, 14th, 15th, 16th, 18th and 19th stages and all the out-of-competition samples were screened for erythropoietin and hydroxyethyl starch (HES).

This type of screening was carried out on a total of 100 samples (70.4% of all the samples taken during the Tour), which marks an increase compared with the last few years (82 samples in 2002 and 72 in 2001).

All the samples were also screened for glucocorticosteroids.

The timetable for taking samples was drawn up by the French Ministry of Sport and the LNDD in cooperation with the UCI Anti-doping Commission. Either the Ministry or the UCI gave the laboratory the order to carry out the EPO analyses on the samples.

During the Tour de France the time taken for the results to be issued to the WADA IO team was on average around 66 and 72 hours after receipt of the samples at the laboratory for normal and EPO screening procedures respectively.

The time for issuing the results for normal procedures was around 100 hours for samples taken during the 12th and 14th stages. It must be pointed out that samples arrived at the laboratory every day, at least 14 hours after the in-competition tests had been completed.

Tables 1 and 2 show all the reports indicating the presence of doping agents.

Table 1

Reports indicating the presence of doping agents other than erythropoietin

| Substances | Number of samples | Comments on concentrations |
|-------------------------|--------------------------|--|
| Triamcinolone acetonide | 28 | Median – 6.0 ng / ml Variation – 1.0 ng / ml – 19 ng / ml |
| Betamethasone | 6 | Median – 26 ng / ml Variation – 1 ng / ml -37 ng / ml |
| Salbutamol | 6 | Median – 143 ng / ml Variation – 87 ng / ml -449 ng / ml |
| Dexamethasone | 3 | Median – 10 ng / ml Variation – 8 ng / ml -15 ng / ml |
| Caffeine | 1 | 10.7 µg / ml |
| Terbutaline | 1 | |
| Lidocaine | 1 | |
| Total: | 46 | |

Table 2

Reports indicating the presence of recombinant erythropoietin or with anomalies

| Classification | Number of samples | Comments |
|---|--------------------------|---|
| Presence of recombinant erythropoietin | 1 | |
| Undetectable recombinant erythropoietin | 15 | Camera intensity below 10,000 LAU |
| Unclassifiable recombinant erythropoietin | 4 | Electrophoretic migration between 48% and 65%, between 65% and 85% for NESP and for epoietin alfa and beta respectively |
| Total: | 20 | |

Looking at Table 2, it should be pointed out that 20% of the test results for the samples analysed for EPO had anomalies and that the only positive case was recorded in a sample taken out-of-competition.

The IO team has to emphasise the quality of the LNDD in terms of management, quality control system, facilities, staff and equipment. It would like to thank the laboratory's managers for their due cooperation throughout the whole of the Tour de France.

RECOMMENDATIONS

The IO team would like to make a few constructive recommendations with a view to optimize the services provided by the LNDD during the Tour de France:

- *The laboratory should reduce the time taken to issue results during the Tour de France. In a competition organised in stages like the Tour de France, a delay in announcing a positive result can allow a rider who has taken drugs to distort the competition results for a few days.*

To achieve this aim, the IO team recommends that the laboratory increase its working hours, by operating extra hours at night and during the weekend and that samples are transported at between 0°C and 10°C to facilitate the start of the technical procedures carried out in the laboratory.

- *The laboratory should have a system for receiving the samples 24 hours a day, which would facilitate the security system protecting the samples and the start of the technical procedures.*
- *The laboratory's security system should be reviewed to eliminate a few weak spots.*
- *The laboratory should enter in the report the codes for the technical procedures used in the analyses to make the information clearer for its customers.*

8. HANDLING THE RESULTS

The IO team would like to say at this point that, in addition to the analysis reports from the LNDD, it also received copies of the reports with the concealed identities of the riders from the doctor taking the samples, because the French law does not allow them to be disclosed.

The results were handled during the Tour de France by the UCI (UCI Anti-doping control regulations) and by the French Council for the Prevention and Fight against Doping (CPLD) (French Public Health Code – Article L 3612-1).

a. WHILE THE OBSERVERS WERE PRESENT

During this period the IO team received a copy of the reports either from the doctor taking the samples or the UCI Medical Inspector the day after each test, in competition or out of competition. The observers have nothing to report during this period.

b. AFTER THE OBSERVERS' DEPARTURE

During this period the President of the IO team received copies of the reports at his confidential fax number in Lisbon or by post.

On 25th July at 1440 (Paris time), the President of the IO team received a fax of analysis report no. 117/07-EPO, which confirmed the presence of recombinant erythropoietin in a sample taken on 18th July during an out-of-competition control. Twenty minutes later the President of the IO team received a telephone call from the President of the UCI Anti-doping Commission, who provided the same information and said that the UCI Medical Inspector was going to inform the rider and his cycling manager after the final stage of the day.

On 1st August the President of the IO team received the report on the B sample analysis carried out at UCI's request on 28th July, which confirmed the presence of recombinant erythropoietin.

On 4th August the President of the IO team requested additional information from the President of the UCI Anti-doping Commission concerning the disciplinary procedures applied in the event of a positive test, as well as information about the existence of other disciplinary procedures concerning positive results obtained by the LNDD, relating to samples taken during the Tour de France (copy of fax in Appendix IV).

On 6th and 7th August the President of the IO team received faxes (in Appendices V and VI) from the President of the UCI Anti-doping Commission informing him that the positive result case was handled in accordance with Articles 174 to 183 of the UCI Anti-doping regulations.

The UCI received the result of the B sample analysis after the final day of the Tour de France and for this reason, it was not possible to apply the principles described in Article 183 of the UCI Anti-doping regulations i.e. to exclude the rider from the race. The case was handed over to the rider's national federation, in accordance with Article 113 of the UCI Anti-doping regulations for it to apply disciplinary procedures, which must be completed within one month of the time limit set for the dispatch of the summons.

In a fax of 6th August the President of the Anti-doping Commission advised the observers that all the other positive cases were examined by the UCI Anti-doping commission, which decided that all these cases were justified on medical grounds. In the case of treatments taken during the Tour de France, all these treatments had been prescribed with the cooperation of the UCI's medical experts and were entered in the riders' health booklets.

The IO team reviewed the drugs declared in the reports and noted that in 71.8% of the samples taken the riders had declared that they had taken a drug. In 60.6% of the samples taken, glucocorticosteroids were administered and in 27.5% of cases beta-2-agonists were used.

The IO team confirmed that, in spite of the information from the UCI Anti-doping Commission about the existence of justified medical grounds in every case where there was a positive result for glucocorticosteroids, the timescales between the date the sample was taken and the date entered in the health booklet when the substance was administered were extremely large.

The IO team calculated these differences in twenty of the twenty-eight positive reports with triamcinolone acetonide, which was 37 days on average, with a variation between 8 and 57 days. In five cases the difference was more than 45 days. The IO team could find no reliable scientific data which could support a urinary excretion time of this duration.

On two occasions the IO team did not find that there was any medical justification concerning the cases showing a positive result for glucocorticosteroids:

- one positive result with triamcinolone acetonide with a medical declaration specifying Betamethasone by infiltration;
- one positive result with Betamethasone (30 ng/ml) with a medical declaration specifying cutaneous application of Betamethasone, the last occasion being 39 days before the test.

On the 18th August the President of the IO team requested additional information from the General Secretary of the CPLD concerning the disciplinary procedures applied with regard to the positive results received from the LNDD, relating to the samples taken during the Tour de France (copy of fax in Appendix VII).

On the same day the President of the IO team received a fax from the General Secretary of the CPLD (in Appendix VIII), informing him that, apart from the cases involving EPO, where proceedings were already under way, the President of the CPLD sent an initial letter to several riders to ensure that a "proof of a medical prescription based on justified therapeutic grounds" is sent to the CPLD.

RECOMMENDATIONS

The IO team recommends the following measures for improving the system for handling results in the Tour de France and other cycling competitions:

- *Results should be handled by an Anti-doping Commission with representatives from the UCI Anti-doping Commission and the CPLD to avoid any conflicts. A single code of regulations should be adopted, in accordance with WADA's World Anti-doping Code.*

- *The UCI should adapt its anti-doping regulations to allow temporary suspension after the result of the B sample analysis has been notified and in accordance with the guidelines applicable to temporary suspensions under the World Anti-doping Code (Article 7.5).*

Any rider who has obtained a positive result from the test carried out during the Tour de France may continue to participate in competitions until the final decision is made by his national federation, which has one or two months (if the national federation has a disciplinary appeal body) to complete the disciplinary procedures.

Until the final decision is made, the rider can help other riders in competitions organised in stages to achieve victories or a good position in the classification. Article 184a of the UCI Anti-doping regulations does not cover every case where temporary suspension proves to be necessary in order to ensure sporting equality.

- *It is necessary to carry out studies into the urinary excretion of glucocorticosteroids following administration of these products by inhalation, local injection, intra-articular injection or other forms of local application to verify the detection time for these substances in urine. We also recommend carrying out studies concerning the metabolism of cortisol in riders in order to detect a temporary or permanent inhibition of its production.*
- *The UCI Anti-doping Commission should be more careful in its analysis of substances and the dates entered in the health booklet when verifying the existence of justified medical grounds if, for instance, the Laboratory has detected a glucocorticosteroid, as specified in point 3 of Article 64 of the UCI's Anti-doping regulations.*

9. INFORMING AND EDUCATING ATHLETES

The day before the Tour de France started, the ASO organised a session to raise the riders' awareness with regards to doping issues in the reception room at the Paris City Hall. This session was attended by Patrice Clerc (President of the ASO), Jean-Marie Leblanc (Director of the Tour de France), Daniel Baal (Deputy Director of the Tour), Jean-François Pescheux (Competition Director), all the teams and their coaches and the members of the WADA IO team.

The organisers of the Tour, in particular, Jean-Marie Leblanc and Daniel Baal spoke in very clear and firm terms about observing the Tour de France's Code of Ethics and they reminded the riders that zero tolerance was applied in this competition.

Daniel Baal gave some explanations about the anti-doping controls which were to be carried out during the Tour and reminded the riders that the UCI would introduce for the first time during the Tour an analysis for free plasma haemoglobin with a view to detecting any possible use of synthetic haemoglobin. He announced that all the riders would receive the same leaflet as in 2002 entitled "Dopage et Cyclisme - ce que vous devez savoir" (Doping and Cycling - what you need to know), which contained very important and useful information for the riders (in Appendix IX).

Daniel Baal stressed the importance of the Tour de France's Code of Ethics (in Appendix X), which appears in the regulations and in the Tour guide book and was signed by all the teams.

This commitment was included both in the Agreement between the ASO and the International Association of Professional Cycling Groups (AIGCP) and in the Agreement signed with each team before the Tour started.

10. UCI MEDICAL COMMISSION

For the third time the UCI appointed a medical commission for the Tour comprising nine doctors. Based on a rota system, two of them were present at all times during the Tour. This commission's task was to observe, advise on and authorise the administration of drugs, especially those subject to restrictions.

The observers requested from the UCI to be informed of the Medical Commission's activities while they were present at the Tour. However, the observers never received any information about any requests made by any team doctor or rider to the Medical Commission.

The observers want to congratulate the UCI for setting up the Medical Commission.

11. MISCELLANEOUS

The French government has set up a system for importing drugs similar to the system introduced during the 2002 Tour.

This involves, in particular, recommendations about importing drugs, with an emphasis on the importance of professional team doctors having two medicine kits (an emergency kit containing possible doping substances and a backup kit without any doping substances).

It is also recommended that a list is kept up to date of all the drugs contained in the two kits being taken out and put back and that these documents can be shown, particularly during custom controls. The CPLD sent the ASO a document summarising these procedures with the reminder that doctors from foreign teams must declare the activities they intend to carry out when in France during the Tour de France. The observers congratulate the French government for this initiative.

The observers also have to congratulate the ASO for its reaction concerning the doping problematic and for implementing during the last few years measures capable of making a significant contribution to the fight against doping during the Tour de France. These include:

- Changes to the course so that fewer kilometres are covered in total and in the time-trial stages and easier stages with fewer hills.
- Ensuring that there are always two days' rest during the Tour with easier transfers.
- Producing a guide for hoteliers offering advice on the cyclists' food requirements and on measures for ensuring the riders get sufficient rest.

12. CONCLUSIONS

The Tour de France is one of the most important sporting events in the world, with huge media coverage and a considerable financial impact.

This is why the positive and negative aspects of this competition will attract so much media attention and therefore, may have major repercussions from an educational point of view.

The scandal during the 1998 Tour de France brought many changes in the fight against doping in almost every sport and in the world of cycling, in particular.

The changes made are moving in the right direction, based on closer cooperation between the responsible bodies (French Ministry of Sport, CPLD, UCI and the Tour de France's organisation) in developing a strategy to combat doping during the Tour de France. The meeting held before the Tour started, between the responsible bodies and WADA, is a good example to support the statement we have made. Accepting an Independent Observer team from WADA is another example of this positive development.

The anti-doping control system developed during the 2003 Tour de France involved considerable sums of money and sometimes was even excessively demanding (e.g. transporting the case containing the samples by aeroplane). It had weaknesses too, though, which may be highly significant in the pursuit of the ultimate goal – to protect the riders' health and retain the true spirit of sportsmanship, especially for the riders who do not use banned substances or methods.

The observers have no doubts at all about the good intentions of all those people involved in planning and implementing the system, but these small weaknesses may help possible cheaters to get round the system or find solutions they can use to defend their actions.

Procedures for taking samples might well be carried out under ideal conditions, but if there are riders who know for sure that they will not be tested twenty minutes before the finish line or even before they have started (time-trial stages) and have the opportunity to perform some kind of physical manipulation before they reach the doping control station, the system cannot guarantee sporting equality.

In spite of some weaknesses in the anti-doping control system, the observers would like to stress that there were strong, positive points to come out of the 2003 Tour de France:

- The firm tone adopted in the speech made by those responsible for organising the Tour de France about observing the Code of Ethics and the fight against doping.
- The soundness of the UCI's health check system and the important role this system plays in the fight against doping in cycling.
- The strategy developed by France in its fight against doping, especially in the area of legislation and through creating the CPLD, providing a high-quality anti-doping control laboratory and implementing measures to prevent trafficking of doping substances.

Closer cooperation between the bodies responsible for anti-doping controls during the Tour de France and the implementation of WADA's World Anti-doping Code and International Standards will be sufficient to guarantee an ideal system. However, all the measures implemented may not be enough if all the partners involved do not assume their responsibilities, especially with regard to protecting the riders' physiological limits. Furthermore, it is important to note that from now on, the observers will only carry out their mission if they have access to all the required documentation.

13. ACKNOWLEDGEMENTS

The observers would like to thank very sincerely all those who contributed to the success of this mission, in particular, the UCI, French Ministry of Sport, the ASO, CPLD and LNDD.

The President of the team would like to express his sincere gratitude to all the members of the IO team who were present during the 2003 Tour de France, where they demonstrated a high level of competence and availability.

14. MEMBERS OF THE INDEPENDENT OBSERVERS TEAM

- Prof. Dr. Luis Horta – Medical expert (POR)

President of the Independent Observers

Medical Doctor with a specialization in sports medicine, Director of the Lisbon Anti-doping Laboratory

- Dr. Anik Sax – Medical expert (LUX)

Independent Observer

Medical Doctor with a specialization in sports medicine, Department Head at the Institute of Sports Medicine in Luxembourg

- Ms. Jennifer Ebermann – Doping Control Expert (GER)

IO Programme Manager/Independent Observer

Manager, WADA

15. APPENDICES

Appendix 1: Procedure for determining blood parameters (UCI)



Juillet 03

Procédure de l'examen médical pour la détermination des paramètres sanguins

0. Conditions générales

Le laboratoire accrédité par l'UCI est l'Institut Indépendant qui assume la responsabilité opérationnelle scientifique et médicale globale de la récolte des échantillons sanguins et des paramètres biochimiques nécessaires à l'établissement de valeurs fiables de l'hématocrite.

Le responsable médical du programme est le Professeur Patrice Mangin.

Pour chaque examen l'UCI désigne **un ou plusieurs Inspecteurs Médicaux**.

La procédure se déroule dans un local adapté qui peut être une chambre d'hôtel. Si possible dans l'hôtel où **sont logés les coureurs, pour les prises de sang. L'appareil de mesure est installé dans un endroit pratique pour la remise des résultats.**

1. La prise de sang doit avoir lieu avant tout effort physique et de préférence avant le petit déjeuner.

A cette fin, l'Inspecteur Médical de l'UCI transmet personnellement le formulaire "Notification au coureur" au Directeur Sportif ou au Chef d'Equipe, à défaut au coureur (dans un délai raisonnable).

2. Le coureur désigné doit se présenter

- dans le délai mentionné sur la notification;
- au médecin de l'Institut Indépendant, dans la chambre mentionnée sur la notification.

Il est obligé de déposer sa licence auprès de l'Inspecteur Médical.

3. Le laboratoire désigné s'engage à assurer les prises de sang veineux chez les cyclistes qui lui sont désignés par l'UCI dans les règles de l'art et à effectuer les analyses dans les délais requis.

A cette fin, **le laboratoire** met à disposition l'équipe médicale scientifique et technique nécessaire; notamment pour la prise de sang, un médecin diplômé, familier des problèmes du sport. **Le laboratoire garantit l'expérience du médecin quant à l'exécution de prises de sang successives.**

Le matériel du prélèvement est fourni par **le laboratoire**.

Les tubes sont rendus anonymes au moment du prélèvement.

NB. Sur demande du coureur, la prise de sang peut également être pratiquée par le médecin du G.S., à la condition *sine qua non* qu'il se conforme au protocole établi. La

Procédure de l'examen médical pour la détermination des paramètres sanguins

présence de l'Inspecteur Médical est obligatoire. A défaut ou si l'échantillon de sang n'a pas été prélevé dès le 1^{er} essai, la ponction est effectuée par le médecin de l'Institut.

4. Après les prises de sang, l'équipe **du laboratoire** effectue les analyses requises.

Elles doivent répondre aux critères de qualité reconnus dans la profession.

A cette fin, l'on utilise un appareil « **COULTER PORTABLE DE LA SERIE A^c ● T ou SYSMEX XT-2000I** ».

Pour l'Hb synthétique, l'appareil « **HEMOCUE LOW PLASMA HEMOGLOBINE** » pourra être également employé.

Les résultats obtenus sur place seront communiqués immédiatement sous forme écrite au responsable désigné par l'UCI, soit l'Inspecteur Médical, soit un médecin de la Commission Sécurité et Conditions du Sport.

La totalité des paramètres sanguins sera communiquée au Président de la Commission Sécurité et Conditions du Sport.

5. L'Inspecteur Médical fait mention:

- A. des résultats aux Directeurs Sportifs ou Chefs d'Equipe concernés, en leur restituant la licence des coureurs examinés,
- B. des résultats inacceptables au Président du Collège des Commissaires au moyen du formulaire de l'UCI "Déclaration d'inaptitude",
- C. des valeurs hématocrite aux coureurs selon leurs désirs.

Cette procédure correspond aux conditions de travail idéales au déroulement des contrôles. Les déviations éventuelles ne peuvent donner lieu à des contestations, s'il n'est pas établi qu'elles ont pu influencer la validité des résultats.

Appendix 2: Measurement protocol (UCI)



Juillet 2003

Protocole de mesure de l'hémoglobine plasmatique libre

A. PRISE DE SANG – ECHANTILLONS A ET B

1. Le coureur doit être en position assise.
2. Le garrot ne doit pas être posé trop longtemps inutilement (délai de moins de une minute entre la pose du garrot et l'apparition du sang dans le tube).
3. Deux tubes de 2,7 ml de sang sont prélevés par une ponction unique. Ils sont désignés arbitrairement échantillons A et B. Ils sont étiquetés avec un numéro identique.
Le tube A est roulé au minimum 15 minutes et analysé immédiatement tel que la procédure le décrit ci-dessous dans le point C.
Le tube B est placé dans un flacon numéroté. Le flacon est attribué à l'équipe toute entière. Le numéro du flacon est inscrit sur le formulaire "Contrôle sanguin" sous le point 9.
Cet échantillon peut être utilisé pour une deuxième analyse, en cas de résultat entraînant une déclaration d'inaptitude.
4. Si pour une raison quelconque, le remplissage du deuxième tube présente des difficultés par la même ponction, il sera demandé au coureur s'il désire une deuxième ponction, sinon il admet qu'en cas de résultat entraînant une déclaration d'inaptitude pour l'échantillon A, la deuxième analyse se fera également sur l'échantillon A. Cette condition est acceptée sous le chiffre 11 du formulaire "Contrôle sanguin".
5. En cas de résultat entraînant une déclaration d'inaptitude pour l'échantillon A, le coureur peut demander que l'échantillon B soit ouvert devant lui et analysé suivant les mêmes règles que pour l'échantillon A. Après une contre-expertise, c'est le résultat de l'échantillon B qui sera pris en compte de manière définitive. La demande d'ouverture de l'échantillon B doit être formulée dans un temps raisonnable, après l'annonce du résultat de l'échantillon A, en tenant compte des impératifs de la course et de la qualité des analyses. Ce délai sera discuté le cas échéant entre l'inspecteur médical, le directeur sportif du coureur et le responsable scientifique de l'équipe médicale (cf. art 13.1.055 du règlement UCI).

C. ANALYSE

1. Préparation des sangs et mesures

Les sangs sont roulés pendant 15 minutes au minimum avant l'analyse pour homogénéisation et stabilisation de température.

Après la détermination des valeurs hématiques habituelles (Hct, Hb, réticulocytes), les échantillons seront centrifugés.

2. Coloration du plasma

Protocole de mesure de l'hémoglobine plasmatique libre

La couleur du plasma sera observée. Si le plasma a une **coloration rose/rouge**, l'échantillon sera alors analysé pour l'hémoglobine plasmatique libre, avec les appareils prévus (Coulter, Sysmex ou Hemocue).

Si la valeur d'hémoglobine plasmatique libre est supérieure à **300mg/dl (3g/l)**, on procédera à l'analyse de l'échantillon B.

- **Analyse de l'échantillon B**
 - Le coureur ou son mandataire est alors informé qu'il peut assister, dans un délai défini par l'inspecteur médical, à l'analyse de l'échantillon B.
 - La contre-expertise sera effectuée à l'aide de l'analyseur Coulter ou Sysmex ou Hemocue.
 - **Déroulement de la contre-expertise :**
 - **effectuer deux mesures à l'aide de l'analyseur Coulter ou Sysmex ou Hemocue**
 - **si la valeur est > à 300mg/dl (3g/l) le coureur est déclaré inapte.**

Appendix 3: Information for riders (UCI)



UNION CYCLISTE INTERNATIONALE
COMMISSION SECURITE ET CONDITIONS DU SPORT

Messieurs les coureurs,

Nous aimerions vous donner quelques informations concernant les contrôles sanguins.

Contrôles sanguins

Lors des contrôles sanguins, notamment pendant le Tour de France, les paramètres habituels seront analysés : hématocrite, hémoglobine et réticulocytes.

Si les analyses devaient montrer des profils anormaux (valeurs ou évolutions anormales), on demandera au coureur (ou à son médecin) quelle est l'origine de cette anomalie, et on lui fera savoir qu'il sera davantage contrôlé, car il restera suspect à nos yeux.

Dès cette année, nous utilisons un nouveau protocole pour identifier les coureurs qui ont un profil sanguin non normal.

Il est évident qu'on les soumettra à des contrôles antidopage, pour la recherche de l'EPO ou du NESP.

Hémoglobines synthétiques

Depuis le début de l'année, nous mesurons, lors des contrôles sanguins, un nouveau paramètre : **l'hémoglobine plasmatique libre**. Ce paramètre augmente de façon très importante (plusieurs centaines de fois) en cas d'administration d'hémoglobine synthétique. Comme vous savez, l'Hb synthétique est une substance interdite, et sa commercialisation à plus large échelle est en train de se réaliser. Or, vu qu'elle est encore indécélable lors des contrôles urinaires, il nous fallait trouver une solution pour décourager son éventuelle utilisation, afin de garantir le droit à avoir des compétitions équitables.

Ce paramètre ne peut pas être employé en tant que test antidopage et, par analogie à ce qui se passe avec les valeurs d'hématocrite élevées, un coureur qui aura une valeur d'**hémoglobine plasmatique libre > 300mg/dl (3g/l)** sera donc **déclaré inapte**, et obligé à se soumettre aux investigations hématologiques. L'hémoglobine synthétique, grâce à la détermination de ce paramètre, peut être détectable pendant plusieurs jours.

Nos équipes médicales vont analyser ce paramètre lors de tous les **contrôles sanguins du matin (y compris celui d'aujourd'hui)**, qui se dérouleront de la même façon qu'auparavant (même quantité de sang prélevée).

En restant à votre disposition pour tout renseignement supplémentaire, nous vous souhaitons tout le succès sportif que vous méritez.

Dr Leon Schattenberg
Président Commission Antidopage

Dr Mario Zorzoli
Médecin UCI

CH 1860 Aigle / Suisse
☎ +41 24 468 58 11 fax +41 24 468 58 12
www.uci.ch

Appendix 4: Request for information 04/08/03

LABORATÓRIO DE ANÁLISES E DOPAGEM
Av.ª. Prof. Egas Moniz (Estádio Universitário)
1600 LISBOA - PORTUGAL

TELEFAX Nº.: 21 797 75 29

De: Prof. Doutor Luís Horta
Para: President of the Medical
Commission of UCI
FAX Nº.: 00 31 46 400 85 21

Nº. de Páginas: 1

Data: 04/08/2003

Dear Dr. Leon Schatewberg

As President of the Independent Observer Mission during the Tour de France, I want kindly request information about the development of the disciplinary procedures done in the positive case met in the sample A 190775 and confirmed in the sample B 190775, on 1st of August.

I want also to request if UCI develop any other disciplinary procedures concerning the positive reports delivery by the French Antidoping Laboratory in the samples collected during the Tour de France.

Best regards.



**President of Independent
Observer Mission**

Appendix 5: UCI reply (07/08/03)

7.AUT.2003 16:17

N9535

P.1



INTERNATIONAL CYCLING UNION

CH 1860 Aigle / Switzerland

☎ : +41 24 468 58 11 – Fax : +41 24 468 58 12

FAX MESSAGE

To : Prof Dr. Luis Horta
Fax nbr : +351 21 797 75 29
From : Dr. Leon Schattenberg
Date : 7 August 2003
Ref : Antidoping Services / Lsch / cv
Total pages : 1 (including this one)
Subject : ***Independent Observer Mission***

Dear Dr. Horta,

I would like to add some information to my last correspondence, As regard to the duration of the proceedings and according art. 113 AER: *"The proceedings before the competent body of the licence-holder's national federation must be completed within one month of the time limit set for the dispatch of the summons."*

We will of course keep you informed all along the procedure. All the documents we will get from the national federation shall be sent to you.

Best Regards,

On behalf of the Antidoping Commission,

A handwritten signature in black ink, appearing to be 'L. Schattenberg', written over a horizontal line.

Dr. Leon SCHATTENBERG, President

Appendix 6: UCI reply (06/08/03)

6 AUG 2003 14:49

N2485

P.1



INTERNATIONAL CYCLING UNION
CH 1860 Aigle / Switzerland
☎ : +41 24 468 58 11 – Fax : +41 24 468 58 12

FAX MESSAGE

To : Prof Dr. Luis Horta
Fax nbr : +351 21 797 75 29
From : Dr. Leon Schattenberg
Date : 6 August 2003
Ref : Antidoping Services / Lsch / cv
Total pages : 2 (including this one)
Subject : ***Independent Observer Mission***

Dear Dr. Horta,

I would like to give you the following information as regard to your demand concerning the Tour de France 2003.

The positive case found in the sample A+B 190775 has been and is being managed according to the UCI AER, art. 174 to 183 (Stage races).

The UCI was informed by the laboratory of the B sample result after the Tour de France ended. Therefore, the case has now been transmitted to the rider's national federation for disciplinary procedure.

As far as the corticoids are concerned, the results have been examined by the UCI Antidoping Commission. All cases were justified on medical grounds and were accepted by the Antidoping Commission. For treatments prescribed during the Tour de France, all of them have been prescribed with the cooperation of the UCI medical experts and treatments have been written in the health booklet.

I also invite you to read two articles written by Dr. Gérard Guillaume:

1. Corticothérapie locale et effet systémique (revue de la littérature) publié dans Médecin du Sport ; Gérard Guillaume et Marcel-Francis Kahn ; e-mail address : g-quillaume@wanadoo.fr
2. Intérêts et limite des infiltrations de corticoïdes dans le sport, publié dans journal de traumatologie du sport.

I will try to send you other information and publication as soon as possible.

Best Regards,

On behalf of the Antidoping Commission,



Dr. Leon SCHATTENBERG, President

Appendix 7: Fax to the CPLD (18/08/03)

Presidência do Conselho de Ministros
Secretaria de Estado da Juventude e Desportos


Instituto do Desporto de Portugal
Laboratório de Análises e Dopagem

FAX

| | | |
|-----------------------------------|---|----------------------|
| REFERÊNCIA: (REFERENCE) | | 049/L.A.D./2003 |
| DATA: (DATE) | | 18 DE AGOSTO DE 2003 |
| PARA: (TO) | CPLD MR. PHILIPPE ROUX-COMELI SÉCRÉTAIRE-GENERALE | |
| A/A: | FAX: 00 33 1 4062 77 39 | |
| DE: (FROM) | INSTITUTO DO DESPORTO DE PORTUGAL – LABORATÓRIO DE ANÁLISES E DOPAGEM | |
| N.º PAGINAS: (NUMBER OF PAGES) | 1 | |
| ASSUNTO: | TOUR DE FRANCE | |

Cher Philippe Roux-Comeli,

Comme President de la Mission d' Observateurs Independents de l' AMA dans le Tour de France, j'ai reçu les copies des PV sans l' identification des coureurs et les rapports analytiques du LNDD.

J' ai reçu plusieurs rapports analytiques positives pour glucocorticosteroids. J'ai demande des informations à l' UCI sur la gestion de ces resultats. L' UCI a informé que sa Comission Médicale a décidé que tous les rapports positives avec glucocorticosteroids étions justifié par une declaration médicale dans le carnet de santé.

Je suis entrain de finir le rapport de notre mission et je veux savoir si le CPLD a quelque chose a ajouter a la position de l' UCI.

Salutations.



Luis Horta
President de la Mission O. I. de l' AMA

AV: PROF. EDUAR MONTE (ESTÁDIO UNIVERSITÁRIO) – 1600-190 LISBOA
TEL: (351)21 798 50 73 – FAX: (351)21 797 75 28

E-MAIL: cmd.labor@mail.telepac.pt

Appendix 8: Reply from the CPLD (18/08/03)

| | |
|--|---|
| <p>CONSEIL DE PRÉVENTION ET DE LUTTE CONTRE LE DOPAGE</p> <p>— ♦ —</p> <p>39, rue Saint Dominique 75007 PARIS</p> <p>----</p> <p><i>Le Secrétaire Général</i></p> | <p><i>République Française</i></p> <p>Paris, le 18/08/2003</p> <p>— ♦ —</p> <p>Tél : 01.40.62.76.76 Fax : 01.47.53.75.36</p> <p><u>Caractère</u> : très urgent <input checked="" type="checkbox"/> urgent <input type="checkbox"/> courant <input type="checkbox"/></p> |
|--|---|

EXPEDITEUR : Philippe ROUX COMOLI

**DESTINATAIRE : Dr Luis HORTA, Président de la mission O.I. de
l'AMA**

N° DE FAX : 00.35.1.217977529

Monsieur le Président,

Le Président du Conseil de prévention et de lutte contre le dopage a reçu l'ensemble des procès-verbaux et résultats d'analyse relatifs aux contrôles effectués lors du Tour de France 2003.

Outre le cas portant sur l'EPO dont la procédure est en cours, il a transmis un premier courrier à plusieurs coureurs afin de s'assurer que ceux-ci transmettent au Conseil « la preuve d'une prescription médicale à des fins thérapeutiques justifiées ». L'instruction est donc en cours.

En restant à votre entière disposition pour toute information complémentaire, je vous prie d'agréer, Monsieur le Président, l'expression de mes sentiments les meilleurs.


Philippe ROUX COMOLI

Nombre de pages y compris celle-ci : 1



Introduction

Chaque sportif souhaite améliorer ses performances.

La compétition étant au cœur du sport, surtout lorsqu'il se pratique à un haut niveau, il est légitime de chercher à se dépasser. Toujours plus haut, toujours plus fort !

Pour y parvenir, certains sont prêts à toutes les compromissions, y compris à tricher. Certains sont prêts à tous les sacrifices, y compris à se détruire la santé. Le dopage est un miroir aux alouettes. Il donne au corps l'illusion d'accroître ses capacités. En fait, sous dopage, le corps va artificiellement au-delà de ses limites. Cela n'est pas sans conséquences.

Ce guide vise à vous informer sur les risques liés au dopage pour votre santé. Il a pour objectif de vous montrer que le dopage est loin d'être anodin.

Ce guide est également destiné à vous montrer à quel point l'idée du dopage est éloignée du sport. Faire du sport, c'est d'abord prendre soin de son corps. Ceci est vrai quel que soit le niveau auquel on le pratique.

Parce que vous êtes des professionnels, ce message vous concerne tout particulièrement. Vous pratiquez un sport de très haut niveau et la tentation du dopage peut être très présente. Il vous concerne aussi parce que vous êtes un exemple pour les jeunes. Il importe de leur montrer, en particulier à ceux qui courront dans quelques années sur les routes du Tour de France, que l'on peut être performant sans se dopage. Car se dopage, c'est mettre en péril sa santé : pour vous-même, pour les autres sportifs et pour le sport, refuser le dopage c'est vital !

Qu'est-ce que le dopage ?

Pour un sportif, se doper consiste à consommer des produits interdits ou à recourir à des méthodes prohibées. Dans tous les cas, l'objectif est d'obtenir une amélioration artificielle de ses performances physiques.

Il existe un grand nombre de produits dopants. Schématiquement, on peut les classer en deux grandes catégories : les médicaments et les stupéfiants. Parmi les médicaments, on trouve notamment les bêta-bloquants, les corticoïdes, les anabolisants, les anesthésiques locaux, les diurétiques et l'hormone de croissance.

Un grand nombre de stupéfiants peut être utilisé comme produit dopant. Citons en particulier les stimulants comme la caféine, comme le cannabis, la méphadone, l'héroïne et la morphine, figurent également parmi les stupéfiants.

Tous ces produits ne sont pas totalement interdits. Certains d'entre eux sont à usage restreint. C'est-à-dire qu'ils ne peuvent être utilisés que dans certaines conditions. C'est par exemple le cas des anesthésiques locaux, des corticostéroïdes et des bêta-bloquants.

Par ailleurs, certaines techniques sont utilisées dans le cadre du dopage. Ces méthodes interdites sont le dopage sanguin et les manipulations chimiques, pharmacologique ou physique visant à masquer les substances dopantes dans les échantillons d'urine utilisés lors des contrôles anti-dopage.

Vrai ou Faux ?

Les produits dopants sont dangereux par eux-mêmes.

Faux

C'est la façon de les consommer qui est dangereuse. Certains des produits utilisés pour se doper sont des médicaments très utiles pour soigner des maladies. Ainsi, il faut bien distinguer l'usage de ces produits et l'abus de leur consommation. C'est l'abus qui est dangereux et qui conduit bien souvent à la dépendance.

Quels sont les risques du dopage ?

Le dopage fait principalement peser des risques pour la santé. La consommation excessive de produits dopants entraîne ainsi de nombreux effets nocifs. Ces derniers peuvent survenir rapidement ou, à l'inverse, apparaître longtemps après la prise du produit dopant (jusqu'à plusieurs années après). A chaque produit sont associés des effets nocifs.

Citons quelques exemples :

Les stimulants (caféine, amphétamines...)

Ils peuvent entraîner des troubles cardio-vasculaires (troubles du rythme cardiaque, infarctus du myocarde, accident vasculaire cérébral), des troubles neurologiques et neuro-musculaires (troubles de la coordination, par exemple) et des troubles psychiques (agressivité ou dépression entre autres).

Les stéroïdes anabolisants

Chez l'homme, ils provoquent des atteintes au niveau de la prostate et du foie, une augmentation de la taille des seins, des troubles du fonctionnement des testicules, une production réduite des spermatozoïdes (avec un risque d'infertilité).

Les diurétiques

Ils peuvent provoquer une déshydratation aiguë, des troubles cardio-vasculaires, une fatigue et un état de faiblesse généralisée, ainsi qu'un dysfonctionnement des reins.

Les hormones peptidiques

(EPO, hormones de croissance, DHEA) : les principaux effets indésirables de ces produits sont la survenue d'une insuffisance cardiaque sévère (pouvant entraîner un infarctus du myocarde, voire une mort subite), d'un diabète, de troubles sexuels et de cancers.

Tous les sportifs recourant à des produits dopants ne présentent pas systématiquement l'ensemble de ces troubles. Il existe en fait une variation importante d'une personne à une autre. Certains seront plus sensibles que d'autres aux effets indésirables d'une consommation de substances dopantes, sans que l'on puisse prédire à l'avance le degré de sensibilité de chacun. Mais il n'existe pas d'individu qui soit totalement insensible aux effets néfastes des produits dopants.

Le saviez-vous ? Le saviez-vous ?

On considère que la durée de vie des sportifs qui se dopent est inférieure d'une vingtaine d'années en moyenne à celle de la population générale.

Comment se passer du dopage ?

Le dopage n'est pas une fatalité, même dans le sport de très haut niveau. De très nombreux champions ont connu de grande carrière sans recourir aux produits dopants. Comment ont-ils fait, comment vous pouvez faire ?

En fait, pour pratiquer un sport, quel que soit son niveau, sans user de substance dopante, il n'y a pas de « recette miracle » ! La performance repose sur les principes de base que sont l'entraînement, la récupération, l'hygiène de vie, le suivi médical et le travail d'équipe.



5 conseils pour améliorer vos performances sans dopage

1 L'entraînement : c'est la base de toute pratique sportive. Sans entraînement, on ne peut maintenir son organisme en situation de réaliser l'activité que l'on souhaite pratiquer. On ne peut pas non plus progresser dans ses performances. L'entraînement doit être adapté à chaque personne, en modulant l'intensité, le volume et la répétition des activités physiques.

2 La récupération : elle est tout aussi essentielle. Sinon, l'organisme se fatigue à l'excès et il devient moins performant. La récupération repose sur l'alternance « travail - repos » qui doit respecter le rythme entre l'éveil et le sommeil. Les techniques de relaxation, le stretching (méthode basée sur des étirements) et l'hydratation favorisent la récupération.

3 L'hygiène de vie : pratiquer un sport suppose une hygiène de vie adaptée. Celle-ci nécessite de respecter ses rythmes biologiques. Par ailleurs, chaque sportif sait combien l'alimentation est importante. Il est donc essentiel d'adopter de bonnes habitudes alimentaires.

4 Le suivi médical : il est très important d'être suivi sur le plan médical très régulièrement. Le médecin doit pouvoir ainsi s'assurer de vos aptitudes à votre activité sportive, tout à la fois sur le plan médical, biologique et psychologique. La relation engagée avec le médecin doit permettre de mettre sur pied une véritable « stratégie de santé ».

5 Le travail d'équipe : dans le cyclisme, la performance est le fruit du travail de toute une équipe. Vous le savez, auprès de vous intervient l'entraîneur, le préparateur physique, le kinésithérapeute, le psychologue et le médecin. Il est important que chacun exerce ses talents dans le respect des autres et des règles. C'est comme cela que les équipes gagnent !

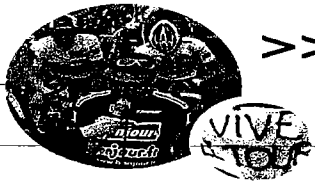
Photos : J.-C. Moreau - B. Bode - ASO



LE TOUR DE FRANCE 02 Dopage et cyclisme ce que vous devez savoir

PACTOR (Générique) BENZIS - VIGNON (du Tour) MONTPELLIER
L'ÉQUIPE (du Tour) MONTPELLIER - L'ÉQUIPE (du Tour) MONTPELLIER

Appendix 10: Tour de France Code of Ethics



>> Le code éthique du Tour de France

Le Tour de France possède en propre des valeurs sans lesquelles il perdrait non seulement sa crédibilité sportive, mais encore son indiscutable fonction culturelle, économique et sociale.

Ces valeurs de référence conservent une signification à travers le temps, tout en prenant en compte les notions de compétition, d'affrontement, de dépassement et en finalité l'objectif majeur de victoire.

Mais il ne peut s'agir ni de n'importe quelle compétition, ni de victoire obtenue à n'importe quel prix.

Comme toutes les activités sportives, comme les autres épreuves cyclistes et davantage encore parce qu'il est la plus prestigieuse de toutes, le Tour de France doit donc s'accompagner :

- De règles précises, dont ni la lettre ni l'esprit ne doivent être transgressés.
- Du respect des officiels chargés de l'application de ces règles et des décisions qu'ils peuvent être amenés à prendre.
- De l'égalité des chances offertes aux concurrents. L'avantage accordé à l'un d'entre eux d'une manière illicite est contraire à l'éthique sportive. C'est en ce sens que le dopage, trop souvent présent dans l'activité sociale ordinaire, est inadmissible en sport.
- D'une action rigoureuse menée contre la tricherie, la corruption et toute forme d'arrangement permettant de vaincre autrement que par les moyens du sport.

L'acceptation de ces principes conditionne l'estime et la popularité qu'accorde au Tour de France un public nourri par une longue période d'exploits et de légende.

Les champions d'aujourd'hui ont en héritage un patrimoine qui ne saurait aller sans les vertus morales qui ont contribué à le forger.

Tout coureur cycliste, quels que soient la place qu'il occupe et le niveau de ses performances, est par conséquent tenu de respecter cette éthique fondamentale. A défaut, il risquerait de conduire son sport vers la perversion et la décadence.





GDC00734

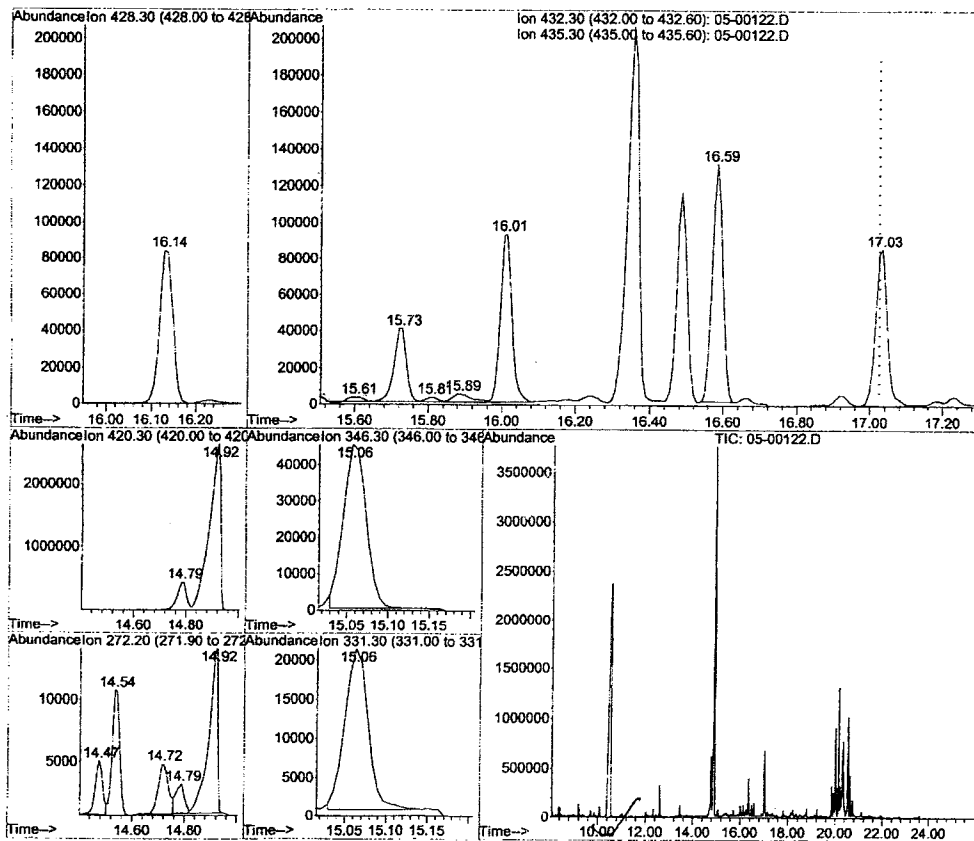
| 995474 | Original Result | Auto | Manual | Zero | Masslynx |
|-----------------|-----------------|-------|--------|-------|----------|
| A Sample | | | | | |
| E-11K | -2.58 | -1.72 | -2.32 | -1.76 | -2.18 |
| A-11K | -3.99 | -3.14 | -3.65 | -2.94 | -3.78 |
| 5B-P | -2.15 | -1.70 | -2.65 | -2.08 | -2.63 |
| 5A-P | -6.14 | -5.65 | -6.95 | -5.55 | -7.22 |
| B Sample | | | | | |
| E-11K | -2.02 | -0.32 | -0.35 | -1.66 | -2.39 |
| A-11K | -3.51 | -1.67 | -1.61 | -2.81 | -4.01 |
| 5B-P | -2.65 | -3.37 | -3.05 | -2.33 | -2.80 |
| 5A-P | -6.39 | -7.61 | -7.19 | -5.58 | -7.03 |

| Blanks | Original Result | Auto | Manual | Zero | Masslynx |
|-----------------|-----------------|-------|--------|-------|----------|
| A Sample | | | | | |
| E-11K | -0.87 | -0.51 | -0.56 | -0.06 | 0.09 |
| A-11K | -0.48 | -0.49 | -0.53 | -0.02 | -0.59 |
| 5B-P | -0.55 | -0.92 | -0.27 | -0.47 | -1.00 |
| 5A-P | -1.59 | -3.65 | -1.87 | -1.46 | -2.45 |
| B Sample | | | | | |
| E-11K | -1.08 | -1.11 | -0.94 | -0.25 | -0.51 |
| A-11K | -0.08 | 0.03 | 0.17 | 0.83 | 0.55 |
| 5B-P | -0.67 | -1.33 | -0.69 | -0.54 | -1.52 |
| 5A-P | -1.60 | -3.45 | -1.89 | -1.24 | -3.66 |

Rapport de quantification
Quantification Testostérone et Épitestostérone
Dernière modification: Mon Nov 15 10:35:39 2004

Fichier : M:\DATA\050120RC\G
Opérateur : CHRISTOPHE
Échantillon : 05-00122 dep apres HRMS
Info. suppl.: LOT P4 # 05-016 dep apres HRMS
Instrument : MS¹²
Vial no : 3
Analyse : 12:21 par P4SIM.M
Quantification: 12:47 par M:\METHODES\P4SIM.M
Multiplicateur: 33.33

| Produit | Signal | TR | Aire | Concentration |
|-------------------|------------|-------|---------|---------------|
| ISTD 2 | m/z 428.30 | 16.14 | 1705309 | |
| Testosterone | m/z 432.30 | 16.59 | 2509863 | 69.7 ng/mL |
| Epitestosterone | m/z 432.30 | 16.01 | 1986949 | 57.3 ng/mL |
| ratio T/E corrigé | | | | 1.38 |
| ISTD 3 | m/z 435.30 | 17.03 | 1857726 | |



Échantillon: 05-00122 dep apres HRMS

Page 1 de 4

PETER HEMMERSBACH, PH.D. WITNESS STATEMENT

I, Peter Hemmersbach, will state as follows:

1. Background

- 1.1. I have been engaged in doping control analysis since 1985 at the Hormone Laboratory at Aker University Hospital in Oslo, Norway. From 1991 I have been head of the IOC-accredited laboratory (from 2004 WADA-accredited) for doping control analysis.
- 1.2. I got my professional education in chemistry from the University in Münster, Germany, with a Dipl. Chemiker (comparable MSc) and Dr. rer. nat. (comparable PhD) degree.
- 1.3. From 2000 I have been appointed as Professor II for pharmaceutical and doping analysis at the University in Oslo, School of Pharmacy, Department for Pharmaceutical Chemistry, Norway.
- 1.4. From 1996 I have been member of the IOC Medical Commission, Subcommission for Doping and Biochemistry in Sport (from 2004 IOC Medical Commission Games Group).
- 1.5. From 2004 I have been member of WADA's Laboratory Committee. This committee is responsible, among other duties, for reviewing and updating the International Standard for Laboratories as well as the Technical Documents.
- 1.6. The proficiency testing of laboratories engaged in doping control has been determined by the IOC (until 2003) and now by WADA. The above mentioned committees provide advice to the decision-making bodies regarding laboratory recognition.
- 1.7. Since 1999 I have been working as a technical assessor for the national accreditation bodies in Norway, Denmark, Germany, Switzerland and Belgium. The audits I took part in were laboratory accreditations according to ISO 17025.
- 1.8. For several audits I have acted as technical assessor for the Swiss Accreditation Service (SAS). A part of the accreditation process evaluates the competence of the scientific staff to select, validate, and control new methods. During these audits I became familiar with the WADA-accredited laboratory in Lausanne. Although my main field of expertise is chromatographic separation methods with mass spectrometric detection (GC/LC-MS), I have had an opportunity to observe the commitment to and implementation of quality management systems, the good organization of the laboratory, the competence of the employees, and the positive attitude and motivation of the whole staff.

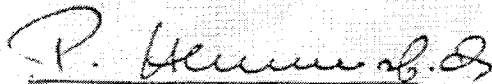
2. ISO and ISL Laboratory Accreditation

- 2.1. The WADA International Standard for Laboratories (ISL) was developed in part as an application of ISO 17025 to the specific needs of the field of doping control. WADA has worked with the International Laboratory Accreditation Cooperation (ILAC) to obtain recognition of the ISL and has trained ILAC

assessors in the use of the ISL in laboratory accreditation of doping control laboratories. Some countries have already adopted the ISL into their assessment visits, which clearly demonstrates the harmony between the ISL and ISO 17025. Thus the ISL is the relevant document for accreditation in doping control, just as the NCCLS EP12-A document referred to by Dr. Strong may be the relevant document for clinical laboratories.

- 2.2. The WADA ISL recognizes the necessity of both quantitative and qualitative (presence or absence) tests in doping control. The WADA Prohibited List prohibits the use of certain substances and methods. Therefore merely the presence of a doping agent, its metabolites or other markers in blood or urine may be proof of a doping offence. Only in the case of substances where endogenous production of the doping agent has to be taken into account is a threshold established. From a perspective of a laboratory measurement, the detection of two populations of red blood cells in a sample is not a physiological condition, and thus this is not a quantitative, threshold measurement.
- 2.3. A qualitative method is as objective as a quantitative approach, as long as the criteria for the presence of a second red blood cell population are adequate.
3. Method validation in qualitative methods
 - 3.1. Method validation is an important process that occurs simultaneously with method development.
 - 3.2. The term validation is used by different groups in different ways. From the laboratory perspective, validation is the confirmation that the method is fit for purpose. Validation is also used in some circles to refer to "proof of concept."
 - 3.3. Validation is carried out in the laboratory performing the test, in this case the LAD. Even if a standard procedure were transferred to the laboratory to follow, the laboratory would be required to carry out its own validation. The extent of the validation is determined by what information is available from previous studies and experience. From reviewing Dr. Saugy's testimony, the LAD did a validation study for the flow cytometry method.
 - 3.4. Validation for a qualitative test differs from a quantitative test. The WADA ISL discussed the validation of these two types of tests separately.
 - 3.5. From the perspective of the ISL and reviewing the written testimony of Dr. Saugy for this hearing, the laboratory appears to have established that no analytical false positives occur by determining that for several hundred known negative samples, the analytical system did not detect any false positive results.
4. Uncertainty in qualitative methods
 - 4.1. The WADA ISL notes that uncertainty, in a quantitative sense, cannot be applied to a qualitative method.

- 4.2. The process of determining uncertainty in qualitative methods is not developed to the extent that uncertainty in quantitative measurements has been.
- 4.3. Uncertainty in a laboratory setting refers to the ability of the analytical process to accurately measure a property of the sample. Some of the studies referred to in Dr. Saugy's testimony would provide an estimate of analytical uncertainty.
- 4.4. It is important in considering the potential for false positive or negative results that the detection capability of the method be considered. The existence of variation below the level of detection of the method would not result in false positives or negatives.



Peter J. Hemmersbach, Ph.D.

12.8.05

Date

Cyclist: Positive test really was negative

[Chicago Final Edition] *Chicago Tribune* - Chicago, Ill.

Author: Philip Hersh, Tribune Olympics reporter

Date: Oct 13, 2006

Section: Sports

Document Types: News Text

Word Count: 506

Taking the case to the public with a PowerPoint presentation and hundreds of pages of documents posted Thursday on his Web site, 2006 Tour de France winner Floyd Landis claimed his positive drug test actually was negative for scientific and secretarial reasons.

Confronted with the same evidence and a related motion for dismissal from Landis' lawyer, the U.S. Anti-Doping Agency's review board still decided Sept. 18 to charge the cyclist with doping based on a positive test for the banned steroid testosterone after the 17th stage of race.

Landis' attorney, Howard Jacobs, wrote off the USADA review as the equivalent of a kangaroo court, challenging its diligence and unwillingness to defy the higher authority of the World Anti-Doping Agency.

"Ask the review board how long they spent [on] it; I'm guessing it wasn't a lot," Jacobs said. "When (WADA chairman) Dick Pound says 'If USADA doesn't proceed, we will,' of course they [will] proceed."

Christiane Ayotte, director of the Montreal anti-doping lab, refuted those accusations. Ayotte also insisted Landis' defense team was inclined to have selected, without context, only pieces of evidence that could support their case from the files of the French laboratory that analyzed Landis' sample.

"WADA and USADA cannot act in a foolish matter," Ayotte said. "They have rules and lawyers. These cases must be based on scientific and legal matters, not political pressure."

The case is headed for an arbitration hearing early next year. Jacobs has asked the hearing be open.

"We are, obviously, not going to participate in a circus, but we have no objection to an open hearing," said USADA general counsel Travis Tygart.

Tygart declined to comment on specifics of the case. WADA spokesman Frederic Donze also declined comment.

The PowerPoint created by Arnie Baker, a retired San Diego physician identified as a longtime Landis coach and adviser, points out errors in recording information on laboratory forms; contends there is evidence of sample degradation and unacceptable variances in scientific measurements that should be sufficient to invalidate the positive result; and questions conclusions drawn from a carbon isotope ratio (CIR) test designed to see whether the testosterone in an athlete's sample is synthetic rather than naturally produced.

"[Landis' defense] must be able to cast doubt on the relevancy of the overall findings," Ayotte said. "A few boo-boos in hundreds of pages of documents is not enough to say the case goes into the wastebasket."

An athlete is presumed to have doped with testosterone if the ratio of two hormones, testosterone and epitestosterone, is greater than 4-to-1. The urine sample is divided into two parts; Landis' "A" sample had a ratio of 11.4-1 and the "B," 11-1.

Baker cited numbers related to contamination and variance in T/E levels that sounded "troubling" to a source familiar with doping control procedures.

Ayotte found it unlikely that degradation could have occurred in the two days between Landis' giving the urine sample and the lab's beginning analysis of it. She had not yet seen enough of the file to comment on measurement issues.

phersh@tribune.com

PRÉSENTATION
DU MAGAZINE

POUR ANNONCER
DANS LE MAGAZINE

jobboom.com

EMPLOIS GÉNÉRAL

EMPLOIS COMPTABILITÉ/
FINANCES

EMPLOIS GÉNIE

EMPLOIS SOUTIEN
ADMINISTRATIF

EMPLOIS TECHNOLOGIES
DE L'INFORMATION

EMPLOIS VENTES/
SERVICE À LA CLIENTÈLE

VOTRE CARRIÈRE


du 15 juin au
15 août 2005

[À la une]

Christiane Ayotte La créativité en science

par Jean-Sébastien Marsan


Directrice du laboratoire de contrôle du dopage de l'Institut national de la recherche scientifique (INRS)-Institut Armand-Frappier, Christiane Ayotte use d'ingéniosité pour démasquer les stéroïdes anabolisants et autres drogues illégales en usage chez les athlètes professionnels.

«Je n'aborde jamais un projet de recherche sans avoir fait un état de la littérature scientifique. À cette étape, ce n'est pas l'intuition qui me caractérise. La créativité intervient dans l'établissement des hypothèses. «La partie créative et prospective de mes tâches, c'est de suivre la littérature scientifique, de regarder ce qui sort de l'industrie pharmaceutique et qui pourrait être détourné [en dopage clinique].»

La créativité dans la science, c'est faire preuve de curiosité multidisciplinaire. «Le défi scientifique en chimie est intéressant, mais ça ne me suffisait pas... J'avais un penchant pour la psychologie, notamment. Je dois penser comme un athlète qui se dope et comprendre le milieu du sport.»

La lutte contre le dopage a aussi des dimensions politiques, économiques, légales, etc., que Christiane Ayotte ne peut ignorer. «Quand des athlètes et de riches avocats américains se battent contre la validité des tests et des contrôles, on a intérêt à être créatif! Et comme ce sont des juges et des avocats qui arbitrent ces causes judiciaires, il faut être capable de vulgariser l'information.»

Dans le merveilleux monde du sport, elle rencontre parfois plus créatif qu'elle : «Pour un résultat positif à un test de testostérone, le sprinter américain Dennis Mitchell avait blâmé la consommation de bière et des relations sexuelles avec sa femme!»

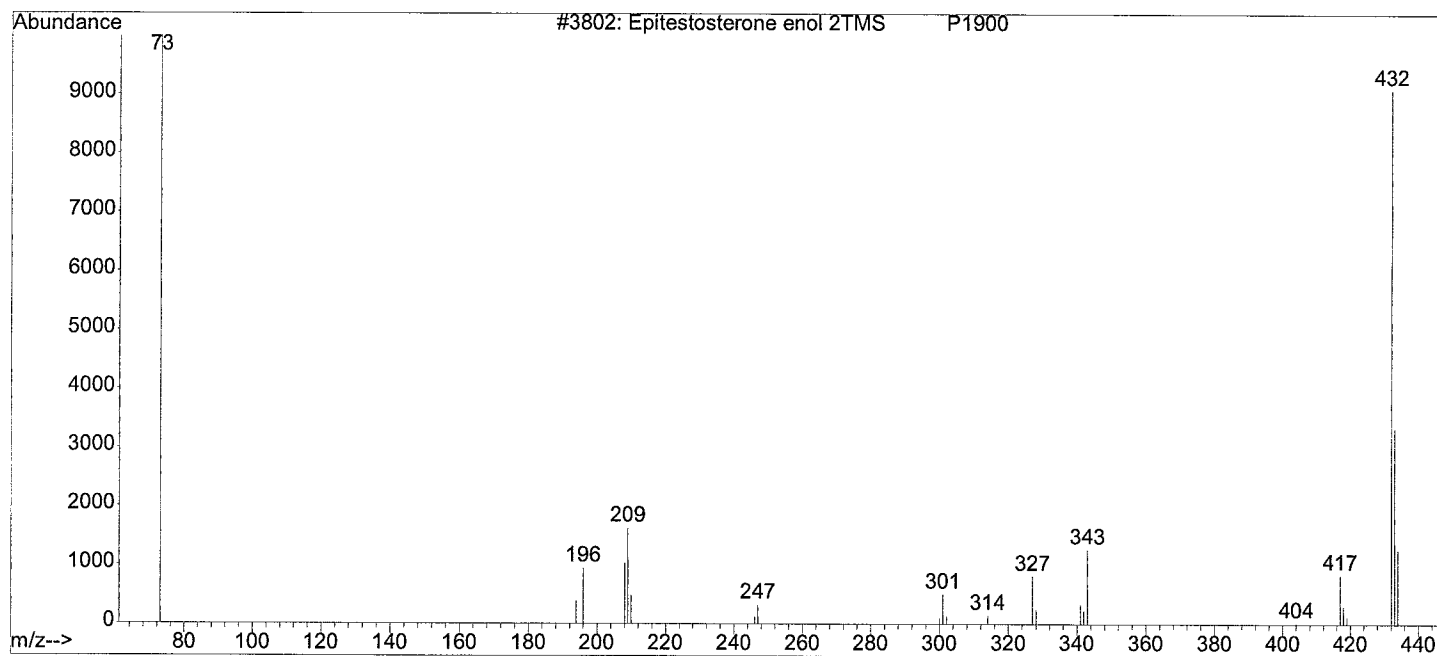
Epitestosterone enol 2TMS

P1900

Entry Number 3802 from C:\DATABASE\PMW_TOX2.L
CAS 000000-00-0
Melting Point 0
Boiling Point 0
Retention Index 2620
Mol Formula C₂₅H₄₄O₂Si₂
Mol Weight 432.287
Company ID Univ-Saar

Miscellaneous Information

Biomolecule %3802



No structure available for 000000-00-0

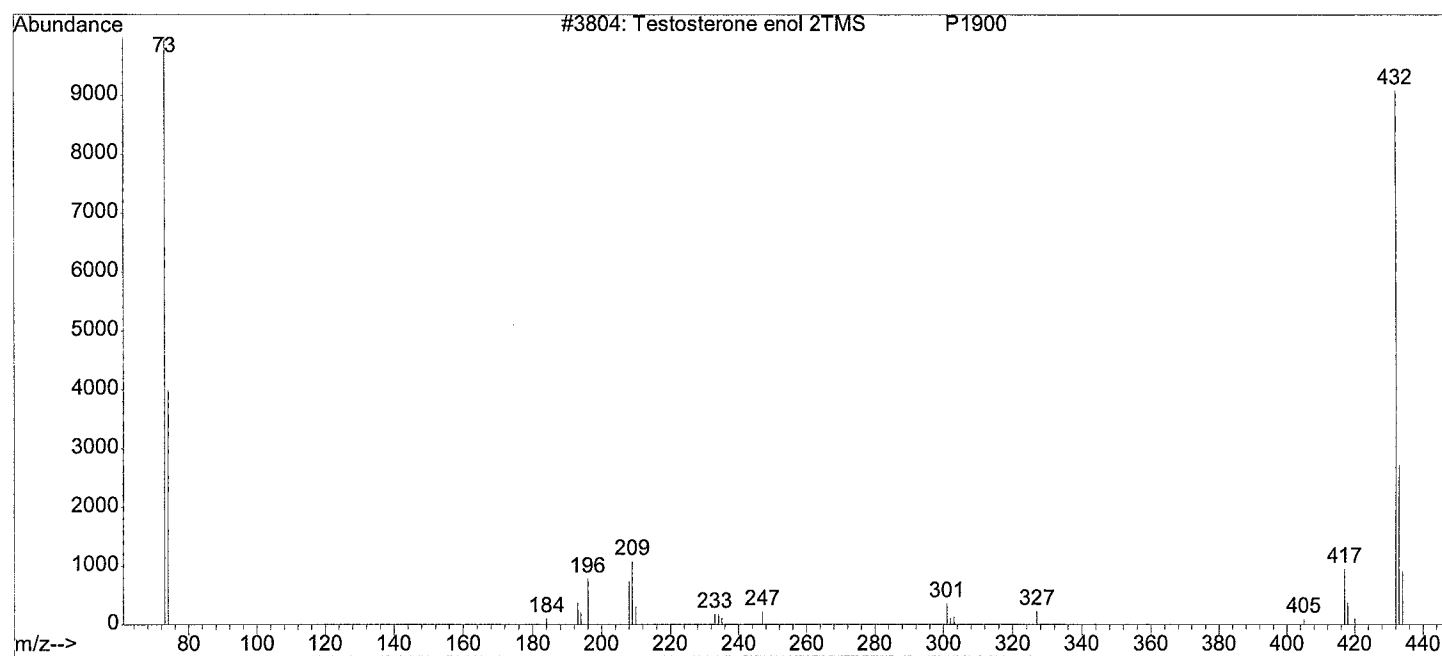
Testosterone enol 2TMS

P1900

Entry Number 3804 from C:\DATABASE\PMW_TOX2.L
CAS 000000-00-0
Melting Point 0
Boiling Point 0
Retention Index 2690
Mol Formula C₂₅H₄₄O₂Si₂
Mol Weight 432.287
Company ID Univ-Saar

Miscellaneous Information

Androgen %3804



No structure available for 000000-00-0

QUANTITATION REPORT FOR COC ON : GC-MS #2

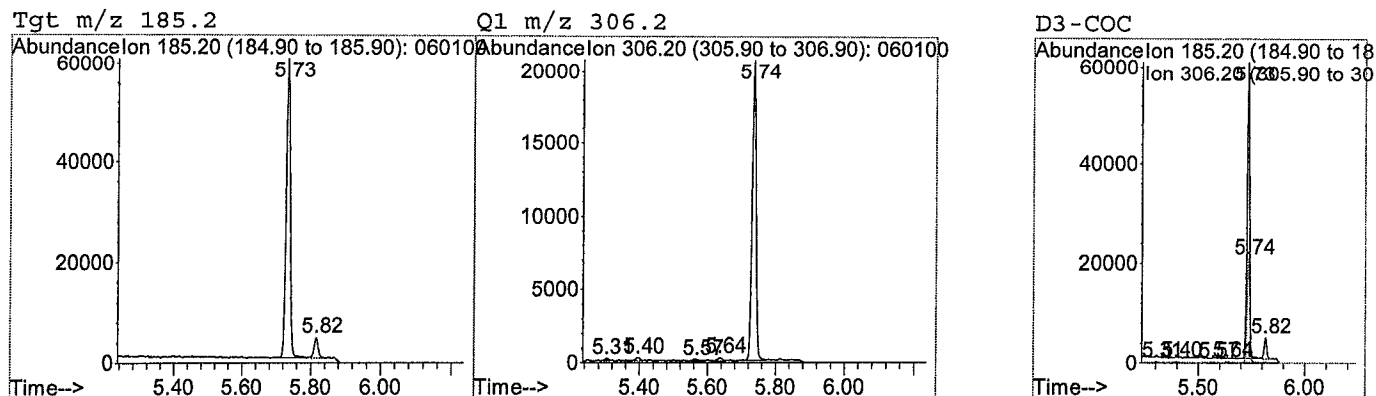
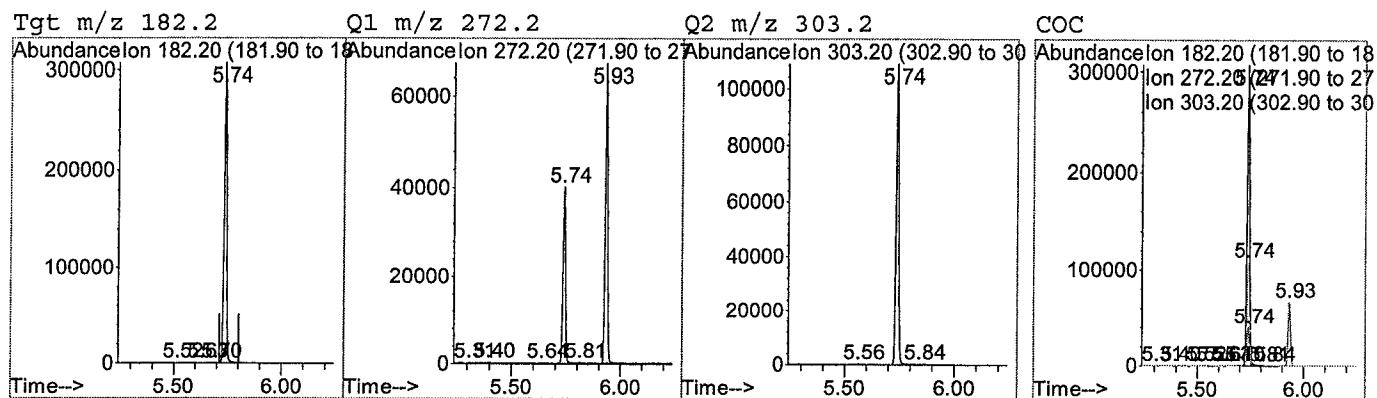
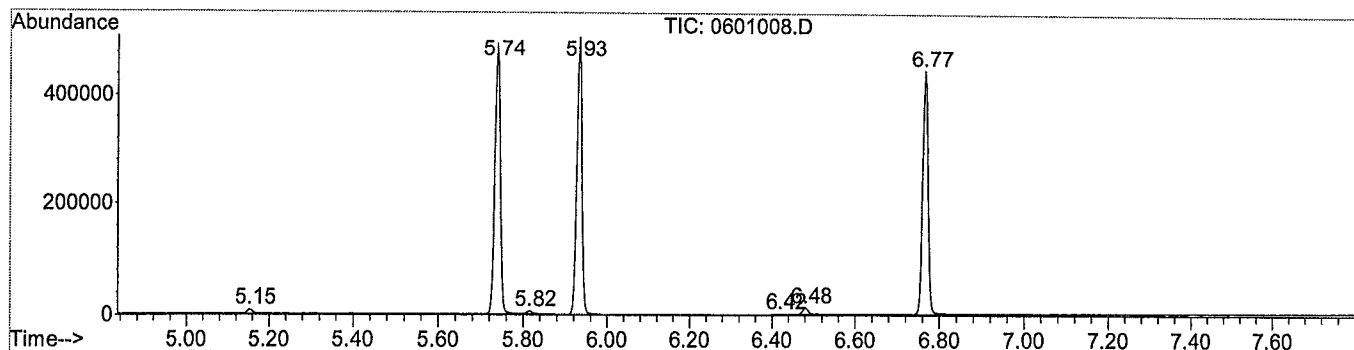
Data File : C:\HPCHEM\1\DATA\14MAY07\0601008.D
Tune File Name : C:\HPCHEM\1\5972\ATUNE.U
Tune Date : 14 May 2007 10:57 am Mult : 1
Acq Method Name : PMCO.C Calib date : 15 May 2007 11:43 am
Sample Name : STD 4: 500
Acquisition date : 14 May 2007 1:09 pm

Retention Time 5.74 COC +/- 1.00% = 5.68 - 5.80 min
Retention Time 5.73 D3-COC +/- 1.00% = 5.68 - 5.79 min
R.R.T. = 1.001 Unknown target ion / ISTD target ion = 5.17

COC => 182.2 = 254424 272.2 = 33074 303.2 = 88382
D3-COC => 185.2 = 49206 306.2 = 16696

COC => 272.2/182.2 = 13.0 +/- 20.0% rel = 10.2 - 15.4
COC => 303.2/182.2 = 34.7 +/- 20.0% rel = 27.5 - 41.3
D3-COC => 306.2/185.2 = 33.9 +/- 20.0% rel = 27.4 - 41.2

Concentration = 511.84 ** COC DETECTED **



COC : RT extraction window from 5.24 to 6.24 min
D3-COC : RT extraction window from 5.24 to 6.24 min

QUANTITATION REPORT FOR BE ON : GC-MS #2

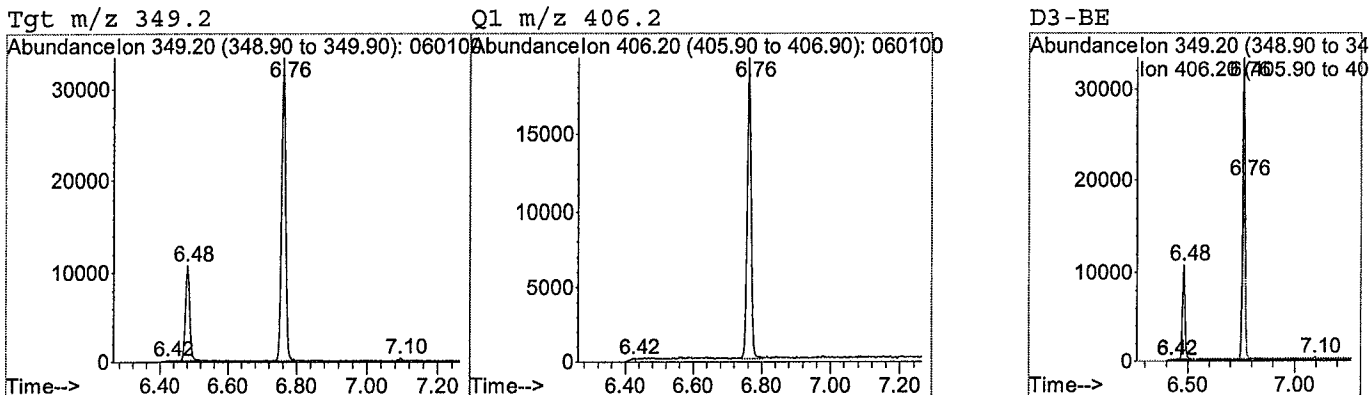
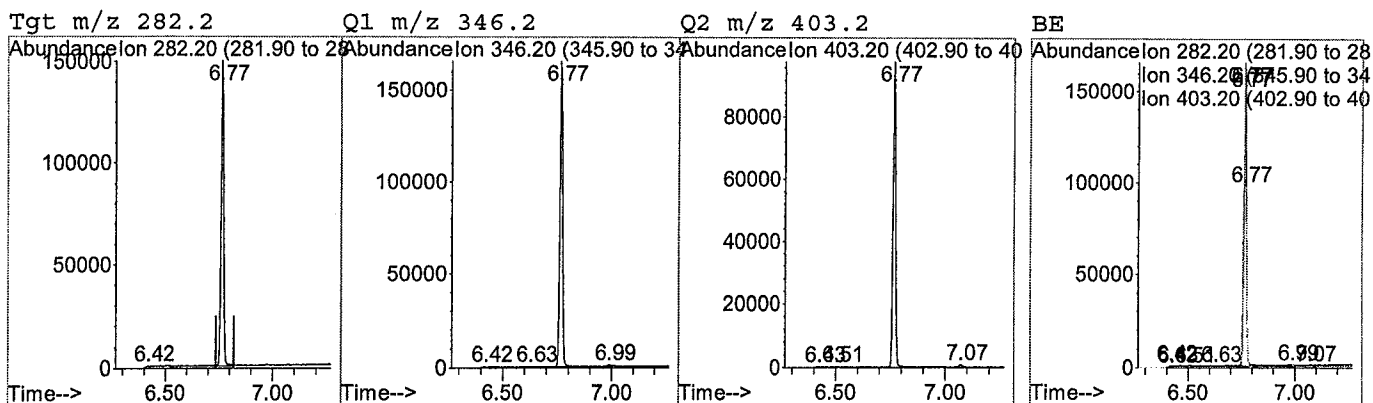
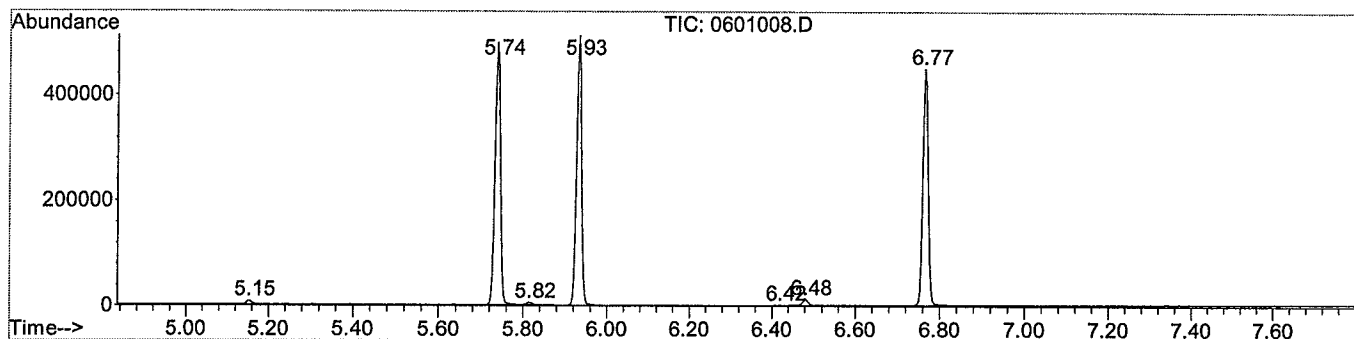
Data File : C:\HPCHEM\1\DATA\14MAY07\0601008.D
Tune File Name : C:\HPCHEM\1\5972\ATUNE.U
Tune Date : 14 May 2007 10:57 am Mult : 1
Acq Method Name : PMCOC.M Calib date : 15 May 2007 11:43 am
Sample Name : STD 4: 500
Acquisition date : 14 May 2007 1:09 pm

Retention Time 6.77 BE +/- 1.00% = 6.70 - 6.83 min
Retention Time 6.76 D3-BE +/- 1.00% = 6.70 - 6.83 min
R.R.T. = 1.001 Unknown target ion / ISTD target ion = 4.59

BE => 282.2 = 124757 346.2 = 140490 403.2 = 81378
D3-BE => 349.2 = 27166 406.2 = 15751

BE => 346.2/282.2 = 112.6 +/- 20.0% rel = 90.7 - 136.1
BE => 403.2/282.2 = 65.2 +/- 20.0% rel = 52.2 - 78.4
D3-BE => 406.2/349.2 = 58.0 +/- 20.0% rel = 44.8 - 67.2

Concentration = 514.17 ** BE DETECTED **



BE : RT extraction window from 6.27 to 7.27 min
D3-BE : RT extraction window from 6.26 to 7.26 min

QUANTITATION REPORT FOR CE ON : GC-MS #2

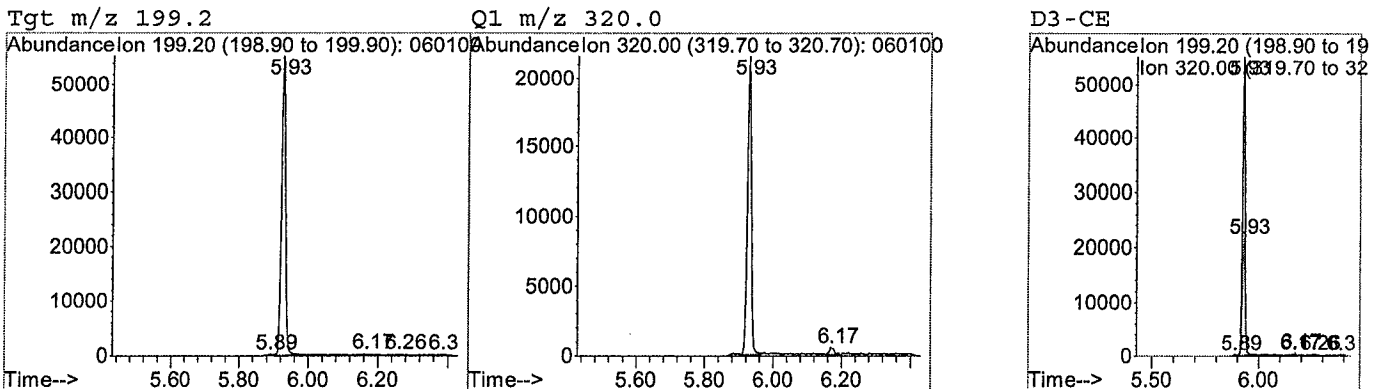
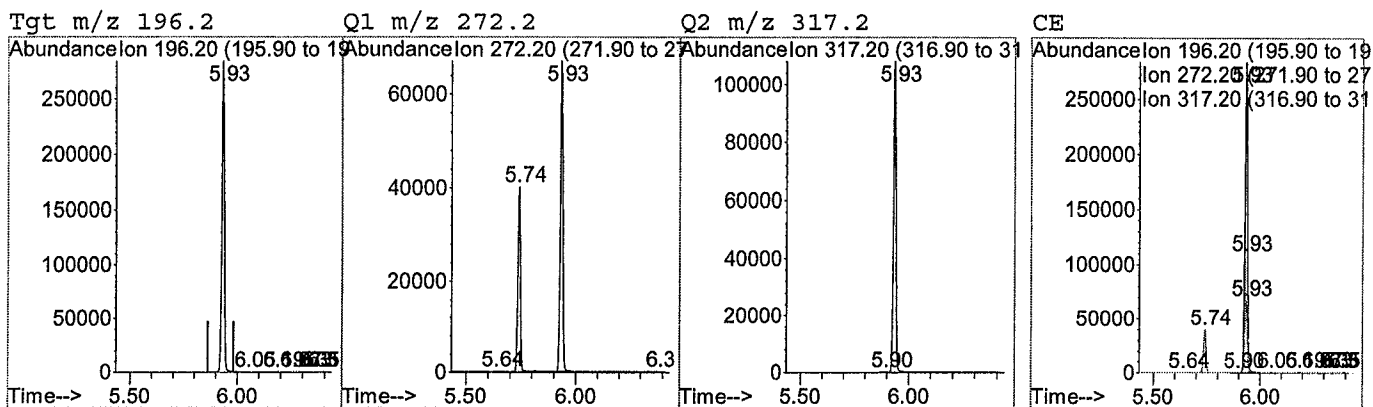
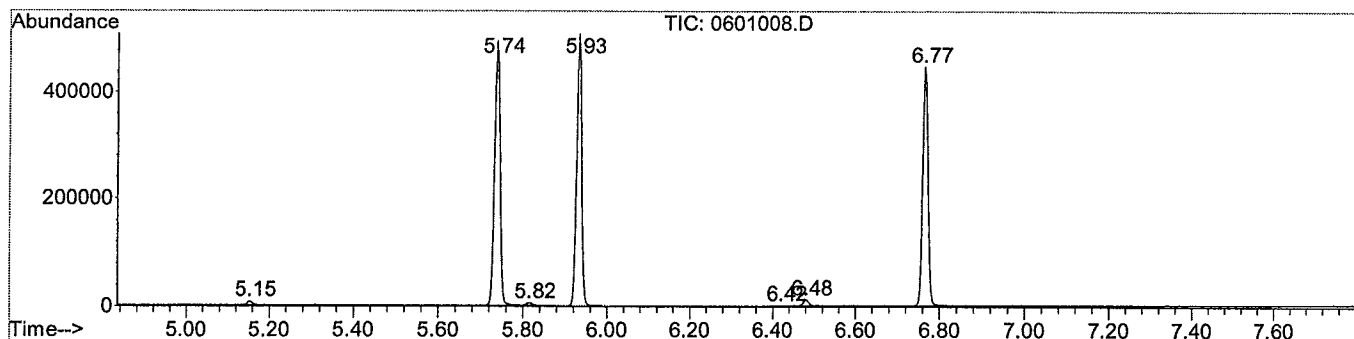
Data File : C:\HPCHEM\1\DATA\14MAY07\0601008.D
Tune File Name : C:\HPCHEM\1\5972\ATUNE.U
Tune Date : 14 May 2007 10:57 am Mult : 1
Acq Method Name : PMCO.C M Calib date : 15 May 2007 11:43 am
Sample Name : STD 4: 500
Acquisition date : 14 May 2007 1:09 pm

Retention Time 5.93 CE +/- 1.00% = 5.88 - 5.99 min
Retention Time 5.93 D3-CE +/- 1.00% = 5.87 - 5.99 min
R.R.T. = 1.001 Unknown target ion / ISTD target ion = 5.74

CE => 196.2 = 263481 272.2 = 53755 317.2 = 87306
D3-CE => 199.2 = 45884 320.0 = 17143

CE => 272.2/196.2 = 20.4 +/- 20.0% rel = 18.5 - 27.7
CE => 317.2/196.2 = 33.1 +/- 20.0% rel = 29.8 - 44.8
D3-CE => 320.0/199.2 = 37.4 +/- 20.0% rel = 29.4 - 44.2

Concentration = 595.28 ** CE DETECTED **

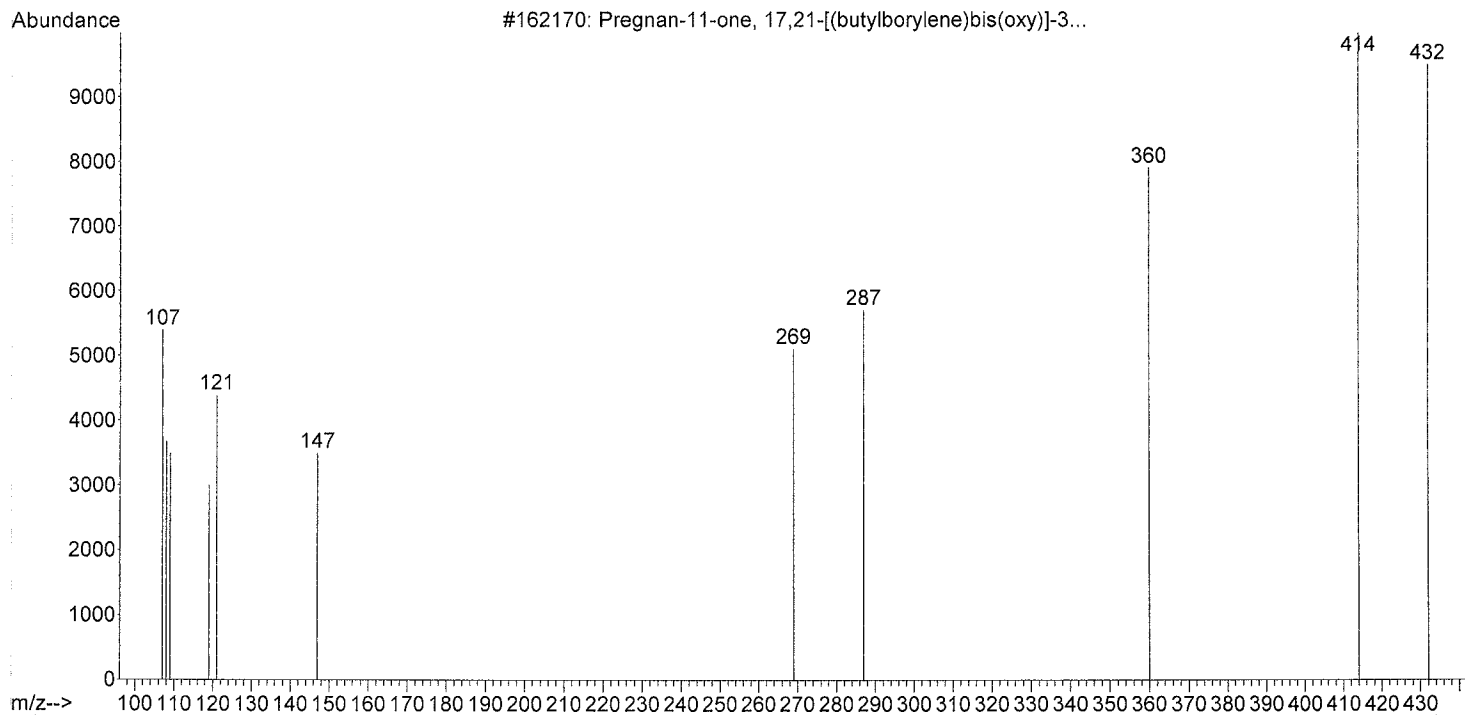


CE : RT extraction window from 5.43 to 6.43 min
D3-CE : RT extraction window from 5.43 to 6.43 min

Pregnan-11-one, 17,21-[(butylborylene)bis(oxy)]-3,20-dihydroxy-, (3.alpha.,5.beta.xy-, (3.alpha.,5.beta.,20S)-

Entry Number 162170 from C:\Database\NIST02.L
CAS 030888-51-8
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C25H41BO5
Mol Weight 432.305
Company ID NIST 2002

Miscellaneous Information
NIST MS# 17500, Seq# M145004



No structure available for 030888-51-8

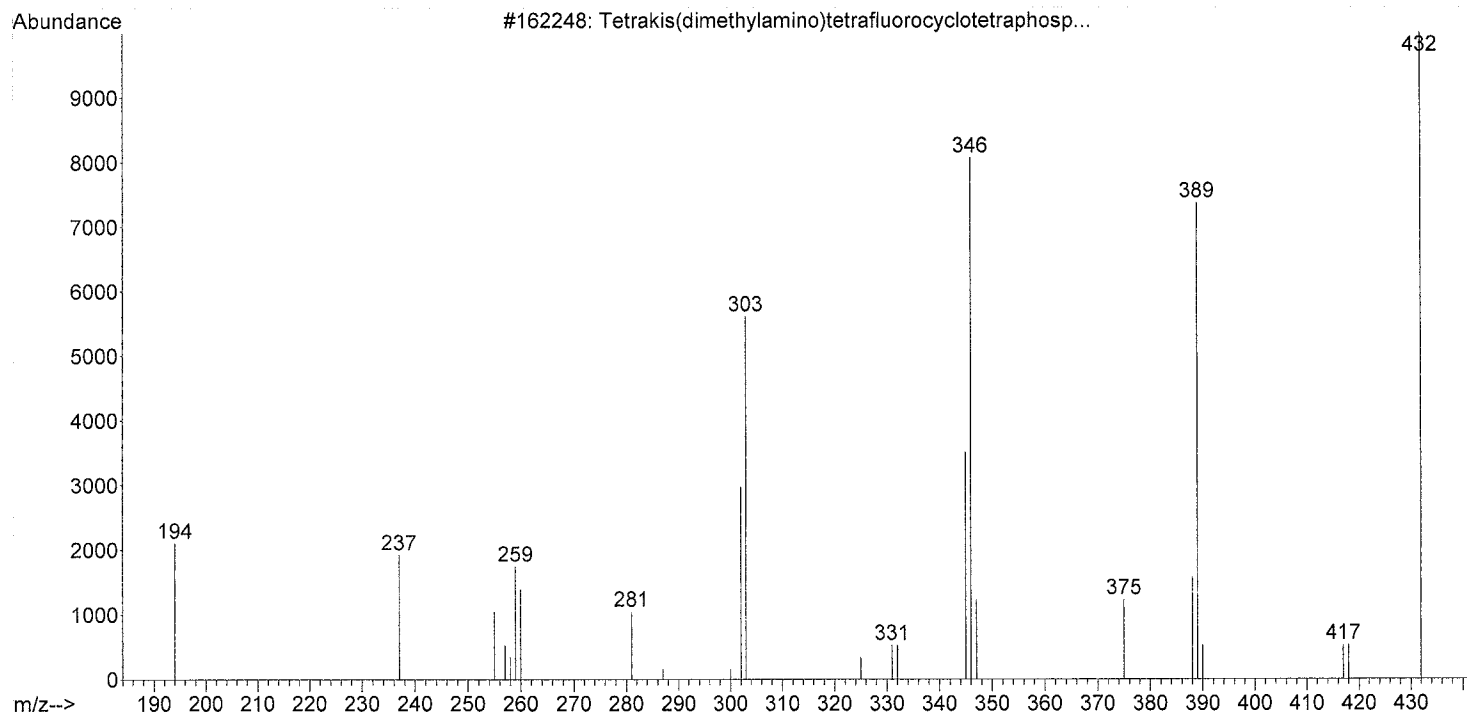
GDC01357.01

Tetrakis(dimethylamino)tetrafluorocyclotetraphosphazene

Entry Number 162248 from C:\Database\NIST02.L
CAS 1000137-65-2
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C₈H₂₄F₄N₈P₄
Mol Weight 432.101
Company ID NIST 2002

Miscellaneous Information

NIST MS# 137652, Seq# M145440, CAS number = 10⁹ + NIST MS#



No structure available for 1000137-65-2

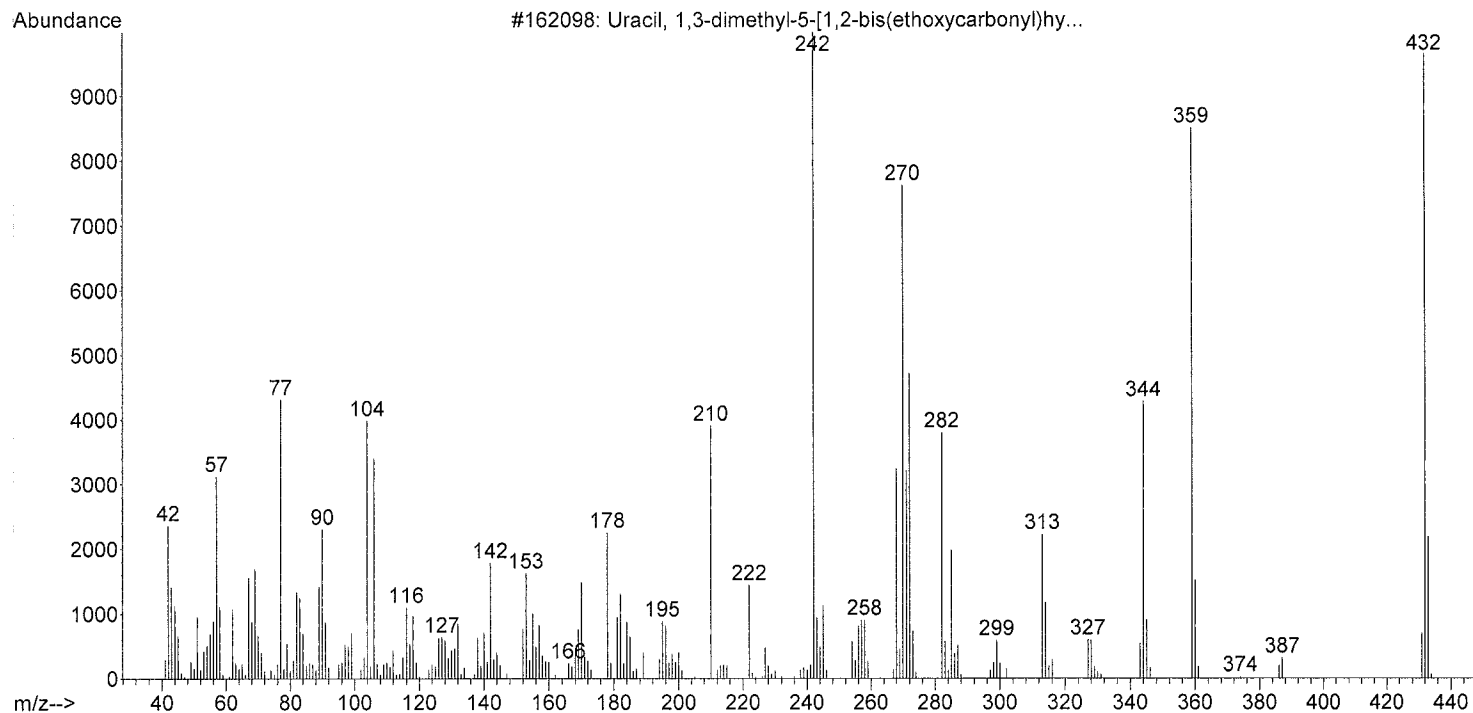
GDC01357.02

Uracil, 1,3-dimethyl-5-[1,2-bis(ethoxycarbonyl)hydrazino]-6-(2-benzylidenehydrazin
(2-benzylidenehydrazino)-

Entry Number 162098 from C:\Database\NIST02.L
CAS 1000255-13-4
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C19H24N6O6
Mol Weight 432.176
Company ID NIST 2002

Miscellaneous Information

NIST MS# 255134, Seq# M124503, CAS number = 10⁹ + NIST MS#



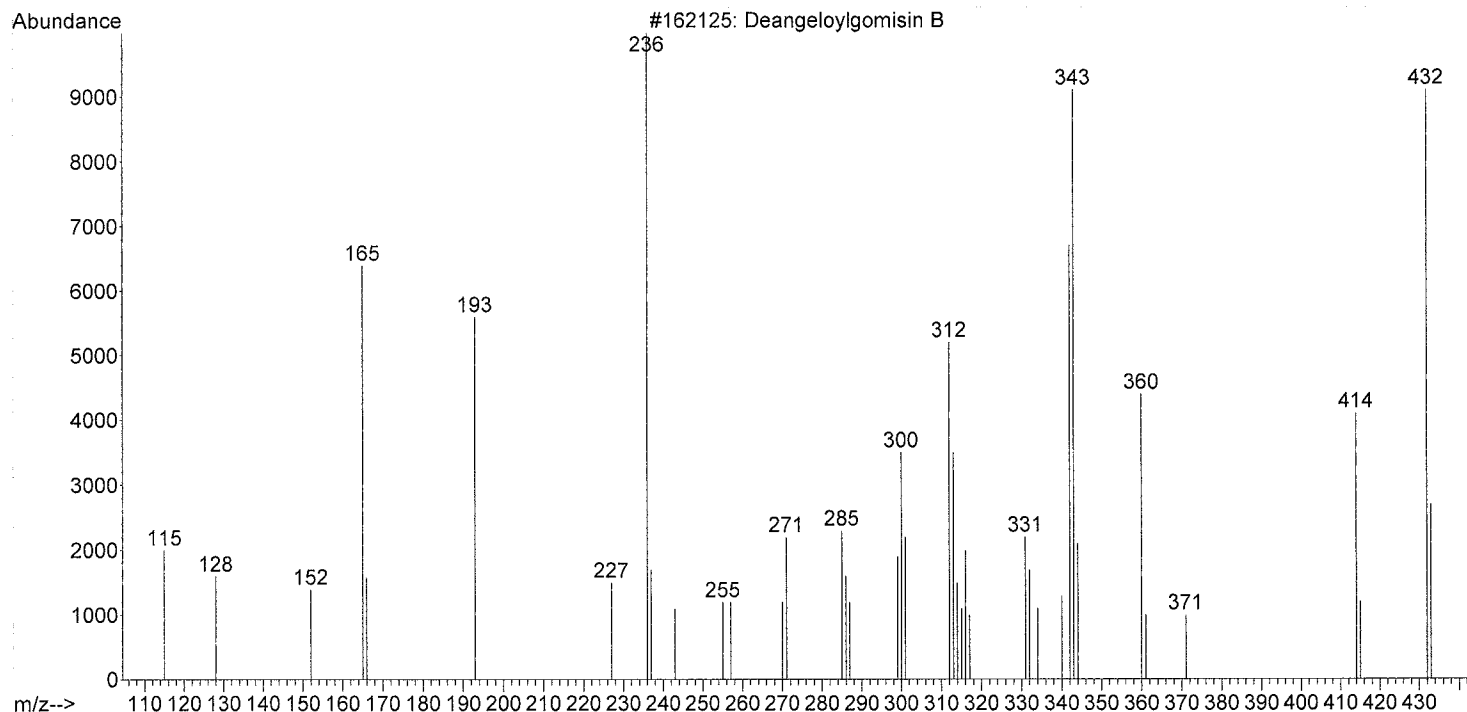
No structure available for 1000255-13-4

GDC01357.03

Deangeloylgomisin B

Entry Number 162125 from C:\Database\NIST02.L
CAS 058546-59-1
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C23H28O8
Mol Weight 432.178
Company ID NIST 2002

Miscellaneous Information
NIST MS# 100539, Seq# M122931



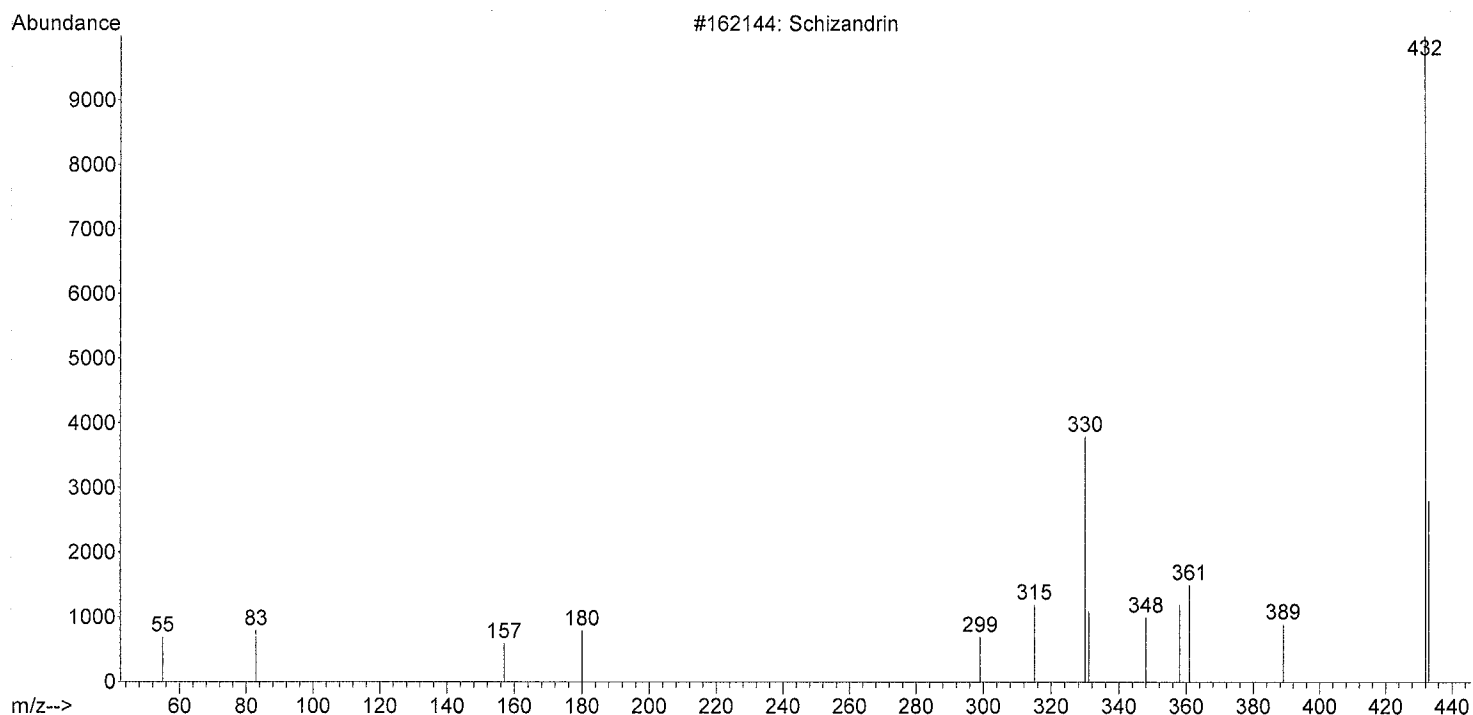
No structure available for 058546-59-1

GDC01357.04

Schizandrin

Entry Number 162144 from C:\Database\NIST02.L
CAS 007432-28-2
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C24H32O7
Mol Weight 432.215
Company ID NIST 2002

Miscellaneous Information
NIST MS# 100535, Seq# M145438



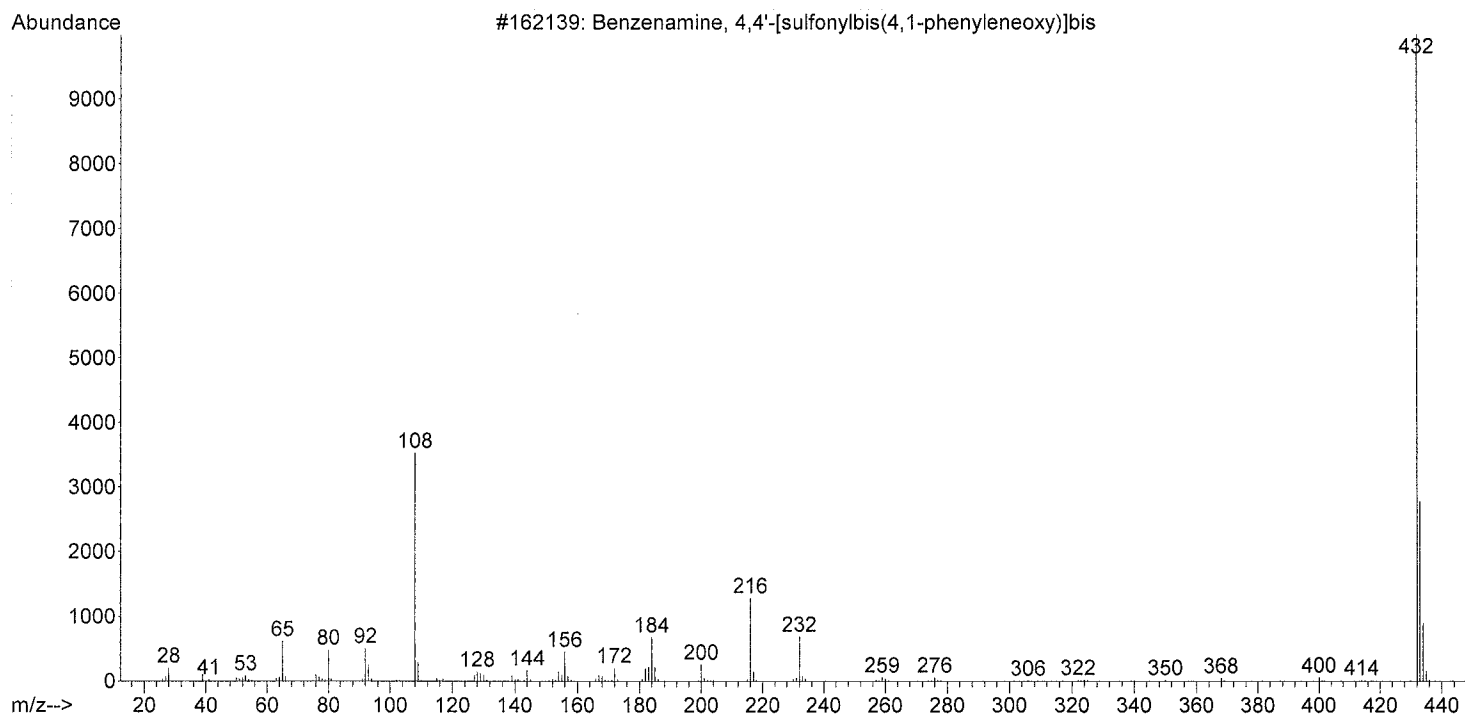
No structure available for 007432-28-2

GDC01357.05

Benzenamine, 4,4'-[sulfonylbis(4,1-phenyleneoxy)]bis-

Entry Number 162139 from C:\Database\NIST02.L
CAS 013080-89-2
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C24H20N2O4S
Mol Weight 432.114
Company ID NIST 2002

Miscellaneous Information
NIST MS# 237533, Seq# M145433



No structure available for 013080-89-2

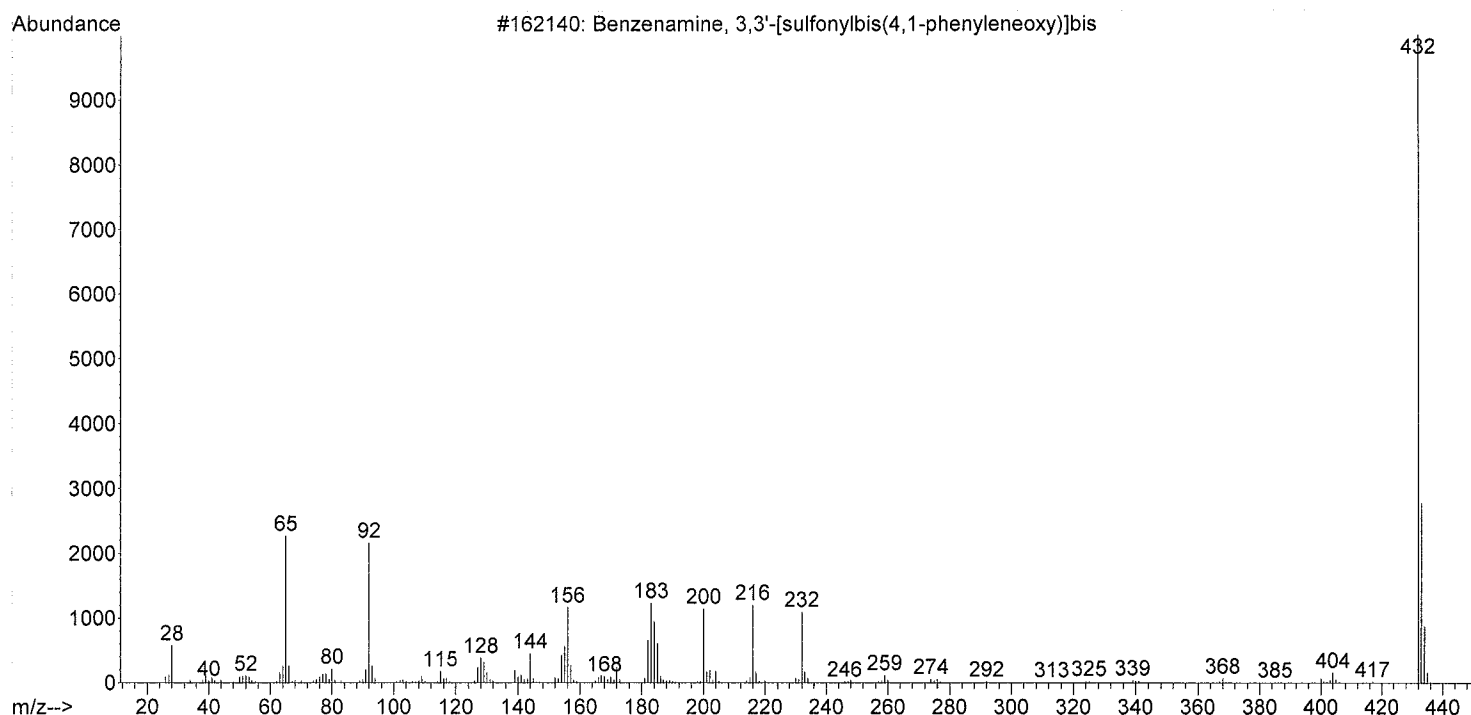
GDC01357.06

Benzenamine, 3,3'-[sulfonylbis(4,1-phenyleneoxy)]bis-

Entry Number 162140 from C:\Database\NIST02.L
CAS 030203-11-3
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C24H20N2O4S
Mol Weight 432.114
Company ID NIST 2002

Miscellaneous Information

NIST MS# 237532, Seq# M145445



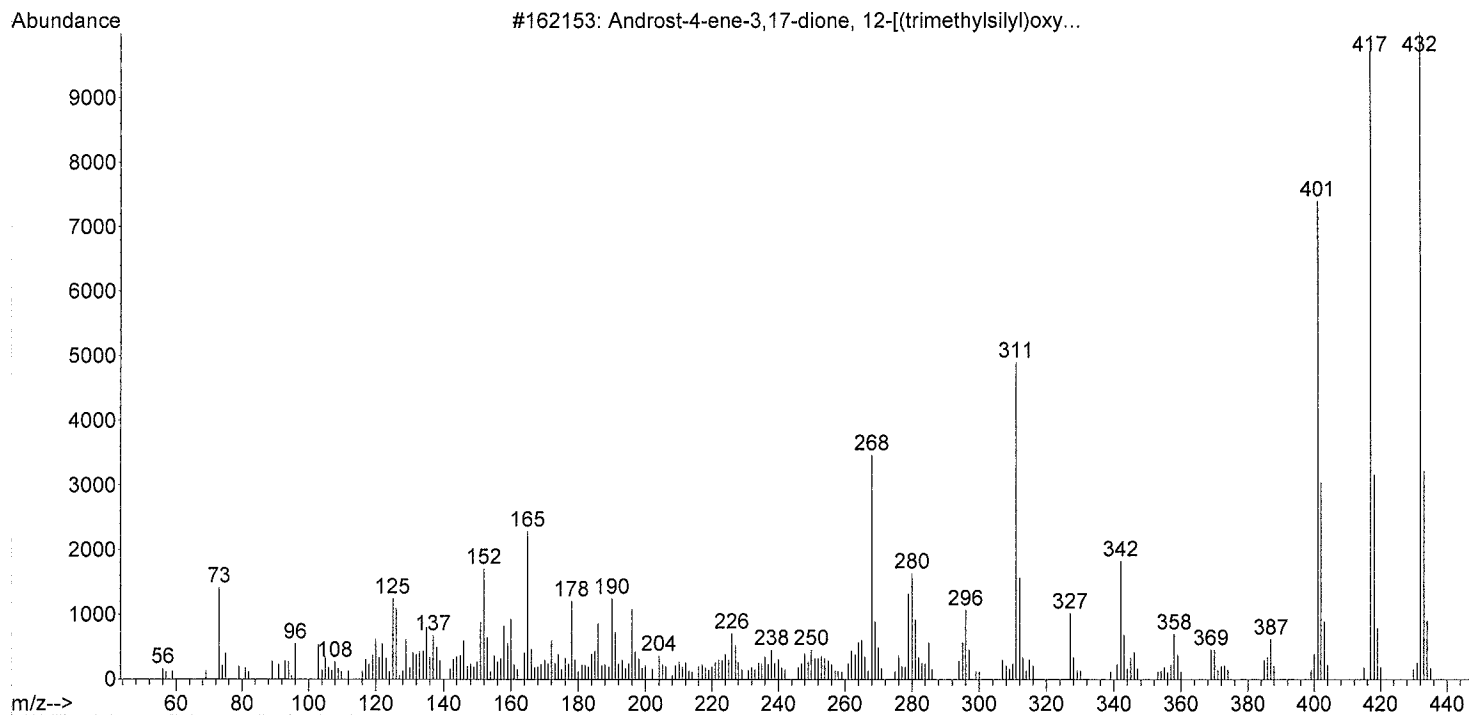
No structure available for 030203-11-3

GDC01357.07

Androst-4-ene-3,17-dione, 12-[(trimethylsilyl)oxy]-, bis(O-methyloxime), (12.beta. ethyloxime), (12.beta.)-

Entry Number 162153 from C:\Database\NIST02.L
CAS 069688-35-3
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C24H40N2O3Si
Mol Weight 432.281
Company ID NIST 2002

Miscellaneous Information
NIST MS# 54916, Seq# M145443



No structure available for 069688-35-3

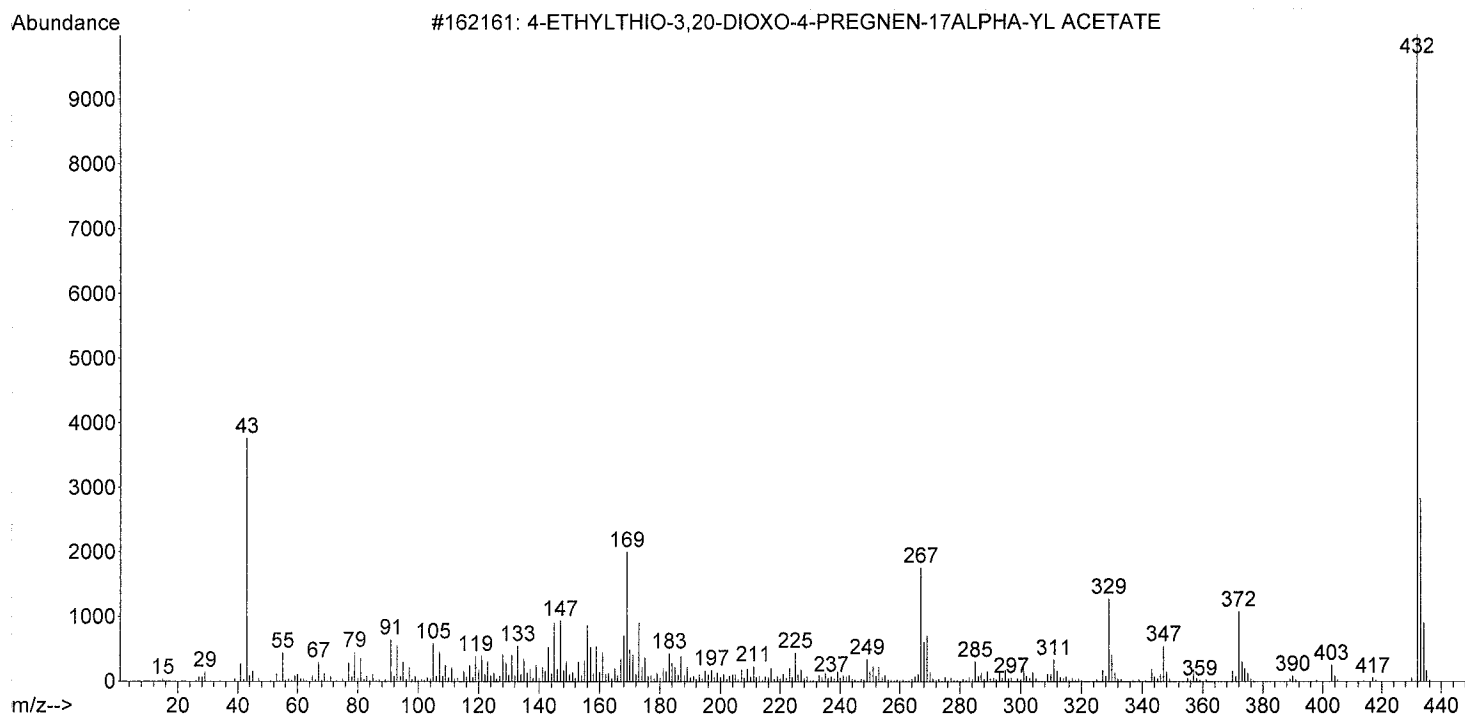
GDC01357.08

4-ETHYLTHIO-3,20-DIOXO-4-PREGNEN-17ALPHA-YL ACETATE

Entry Number 162161 from C:\Database\NIST02.L
CAS 1000244-57-0
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C25H36O4S
Mol Weight 432.233
Company ID NIST 2002

Miscellaneous Information

NIST MS# 244570, Seq# M145431, CAS number = 10⁹ + NIST MS#



No structure available for 1000244-57-0

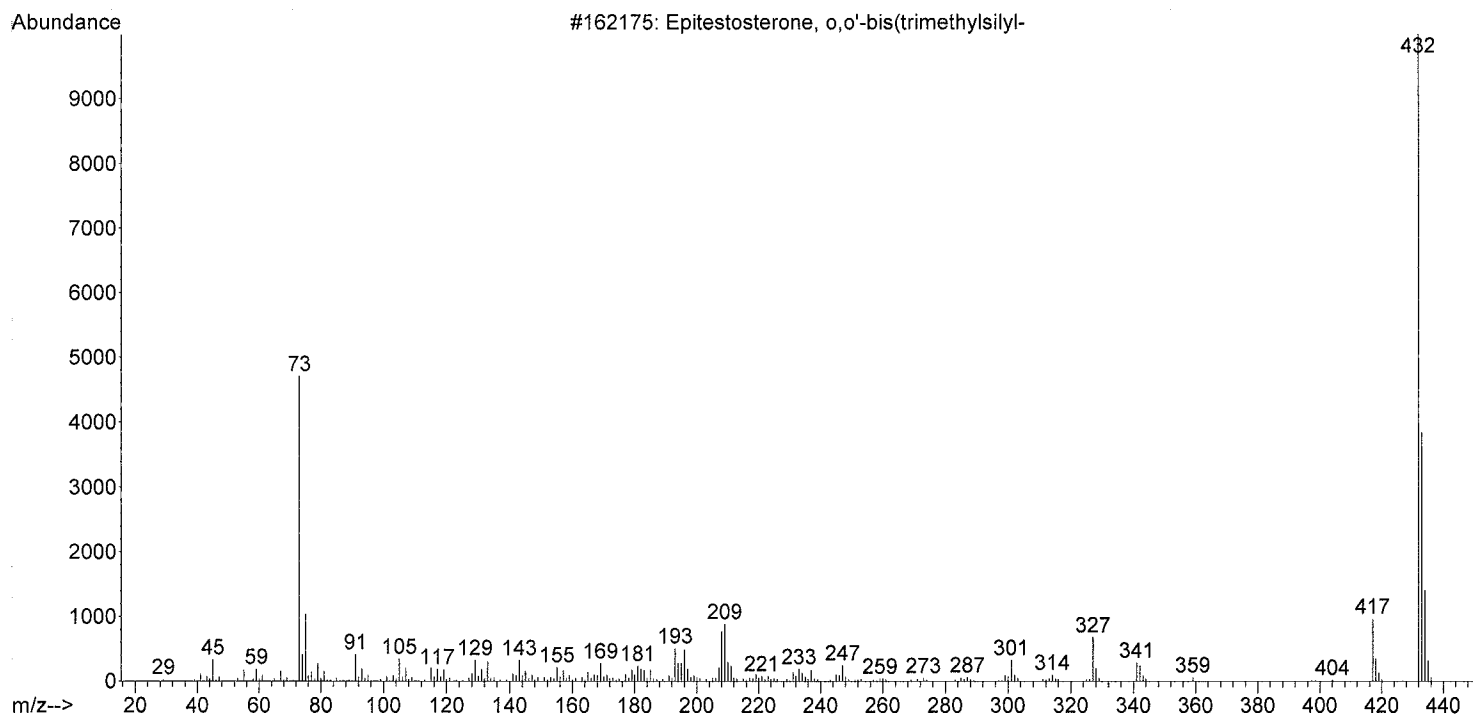
GDC01357.09

Epitestosterone, o,o'-bis(trimethylsilyl)-

Entry Number 162175 from C:\Database\NIST02.L
CAS 1000297-93-7
Melting Point -300
Boiling Point -300
Retention Index 0
Mol Formula C₂₅H₄₄O₂Si₂
Mol Weight 432.288
Company ID NIST 2002

Miscellaneous Information

NIST MS# 297937, Seq# M145432, CAS number = 10⁹ + NIST MS#



No structure available for 1000297-93-7

GDC01357.010

C43-A
Vol. 22 No. 22
Replaces C43-P
Vol. 20 No. 9

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

This document provides guidance on establishing uniform practices necessary to produce quality data for quantitation and identification of a drug or drug metabolite using the GC/MS method; specific quality assurance criteria for maintaining and documenting optional instrument performance are also presented. A guideline for global application developed through the NCCLS consensus process.



NCCLS...

Serving the World's Medical Science Community Through Voluntary Consensus

NCCLS is an international, interdisciplinary, nonprofit, standards-developing, and educational organization that promotes the development and use of voluntary consensus standards and guidelines within the healthcare community. It is recognized worldwide for the application of its unique consensus process in the development of standards and guidelines for patient testing and related healthcare issues. NCCLS is based on the principle that consensus is an effective and cost-effective way to improve patient testing and healthcare services.

In addition to developing and promoting the use of voluntary consensus standards and guidelines, NCCLS provides an open and unbiased forum to address critical issues affecting the quality of patient testing and health care.

PUBLICATIONS

An NCCLS document is published as a standard, guideline, or committee report.

Standard A document developed through the consensus process that clearly identifies specific, essential requirements for materials, methods, or practices for use in an unmodified form. A standard may, in addition, contain discretionary elements, which are clearly identified.

Guideline A document developed through the consensus process describing criteria for a general operating practice, procedure, or material for voluntary use. A guideline may be used as written or modified by the user to fit specific needs.

Report A document that has not been subjected to consensus review and is released by the Board of Directors.

CONSENSUS PROCESS

The NCCLS voluntary consensus process is a protocol establishing formal criteria for:

- the authorization of a project
- the development and open review of documents
- the revision of documents in response to comments by users
- the acceptance of a document as a consensus standard or guideline.

Most NCCLS documents are subject to two levels of consensus—"proposed" and "approved." Depending on

the need for field evaluation or data collection, documents may also be made available for review at an intermediate (i.e., "tentative") consensus level.

Proposed An NCCLS consensus document undergoes the first stage of review by the healthcare community as a proposed standard or guideline. The document should receive a wide and thorough technical review, including an overall review of its scope, approach, and utility, and a line-by-line review of its technical and editorial content.

Tentative A tentative standard or guideline is made available for review and comment only when a recommended method has a well-defined need for a field evaluation or when a recommended protocol requires that specific data be collected. It should be reviewed to ensure its utility.

Approved An approved standard or guideline has achieved consensus within the healthcare community. It should be reviewed to assess the utility of the final document, to ensure attainment of consensus (i.e., that comments on earlier versions have been satisfactorily addressed), and to identify the need for additional consensus documents.

NCCLS standards and guidelines represent a consensus opinion on good practices and reflect the substantial agreement by materially affected, competent, and interested parties obtained by following NCCLS's established consensus procedures. Provisions in NCCLS standards and guidelines may be more or less stringent than applicable regulations. Consequently, conformance to this voluntary consensus document does not relieve the user of responsibility for compliance with applicable regulations.

COMMENTS

The comments of users are essential to the consensus process. Anyone may submit a comment, and all comments are addressed, according to the consensus process, by the NCCLS committee that wrote the document. All comments, including those that result in a change to the document when published at the next consensus level and those that do not result in a change, are responded to by the committee in an appendix to the document. Readers are strongly encouraged to comment in any form and at any time on any NCCLS document. Address comments to the NCCLS Executive Offices, 940 West Valley Road, Suite 1400, Wayne, PA 19087, USA.

VOLUNTEER PARTICIPATION

Healthcare professionals in all specialties are urged to volunteer for participation in NCCLS projects. Please contact the NCCLS Executive Offices for additional information on committee participation.

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

Abstract

NCCLS document C43-A— *Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline* is intended to aid the laboratorian in developing appropriate procedures for the use of GC/MS in confirmation analyses. Its primary objective is to establish uniform practices necessary for producing quality data for quantitation and identification of a drug or drug metabolite. To support the scientific basis of the uniform practices, a brief overview of the techniques is provided. Specific quality assurance criteria for maintaining and documenting optimal instrument performance are presented.

NCCLS. *Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline*. NCCLS document C43-A (ISBN 1-56238-475-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002.

The NCCLS consensus process, which is the mechanism for moving a document through two or more levels of review by the healthcare community, is an ongoing process. Users should expect revised editions of any given document. Because rapid changes in technology may affect the procedures, methods, and protocols in a standard or guideline, users should replace outdated editions with the current editions of NCCLS documents. Current editions are listed in the *NCCLS Catalog*, which is distributed to member organizations, and to nonmembers on request. If your organization is not a member and would like to become one, and to request a copy of the *NCCLS Catalog*, contact the NCCLS Executive Offices. Telephone: 610.688.0100; Fax: 610.688.0700; E-Mail: exoffice@nccls.org; Website: www.nccls.org

C43-A

ISBN 1-56238-475-9

ISSN 0273-3099

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

Volume 22 Number 22

Larry D. Bowers, Ph.D., DABCC, Chairholder

David A. Armbruster, Ph.D., DABCC

Thomas Cairns, Ph.D., DABCC

Major John T. Cody, Ph.D., D-FTCB

Robert Fitzgerald, Ph.D., DABCC

Bruce A. Goldberger, Ph.D., DABFT

David Lewis

Leslie M. Shaw, Ph.D.



GDC01358.5

This publication is protected by copyright. No part of it may be reproduced, stored in a retrieval system, transmitted, or made available in any form or by any means (electronic, mechanical, photocopying, recording, or otherwise) without prior written permission from NCCLS, except as stated below.

NCCLS hereby grants permission to reproduce limited portions of this publication for use in laboratory procedure manuals at a single site, for interlibrary loan, or for use in educational programs provided that multiple copies of such reproduction shall include the following notice, be distributed without charge, and, in no event, contain more than 20% of the document's text.

Reproduced with permission, from NCCLS publication C43-A—*Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline* (ISBN 1-56238-475-9). Copies of the current edition may be obtained from NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

Permission to reproduce or otherwise use the text of this document to an extent that exceeds the exemptions granted here or under the Copyright Law must be obtained from NCCLS by written request. To request such permission, address inquiries to the Executive Director, NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

Copyright ©2002. The National Committee for Clinical Laboratory Standards.

Suggested Citation

(NCCLS. *Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline*. NCCLS document C43-A [ISBN 1-56238-475-9]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002.)

Proposed Guideline

April 2000

Approved Guideline

November 2002

ISBN 1-56238-475-9
ISSN 0273-3099

Committee Membership**Area Committee on Clinical Chemistry and Toxicology**

W. Gregory Miller, Ph.D.
Chairholder

Medical College of Virginia Hospital
Richmond, Virginia

Thomas L. Williams, M.D.
Vice-Chairholder

Methodist Hospital
Omaha, Nebraska

Subcommittee on GC/MS

Larry D. Bowers, Ph.D., DABCC
Chairholder

U.S. Anti-Doping Agency
Colorado Springs, Colorado

David A. Armbruster, Ph.D., DABCC

Abbott Laboratories
Abbott Park, Illinois

Thomas Cairns, Ph.D., D.Sc.

Psychomedics
Culver City, California

Major John T. Cody, Ph.D., D-FTCB

Academy of Health Sciences
Fort Sam Houston, Texas

Robert L. Fitzgerald, Ph.D., DABCC

VA (San Diego) Medical Center/UCSD
San Diego, California

Bruce A. Goldberger, Ph.D., DABFT

University of Florida College of Medicine
Gainesville, Florida

David Lewis

Signature Science
Austin, Texas

Leslie M. Shaw, Ph.D.

University of Pennsylvania
Philadelphia, Pennsylvania

Advisors

Bob Brittain

Varian Associates
Walnut Creek, California

Siu C. Chan, Ph.D.

University of Calgary
Calgary, Alberta, Canada

Yeshpal S. Chauhan, Ph.D.

Scarsdale, New York

John J. Fenton, Ph.D.

West Chester University
West Chester, Pennsylvania

Benjamin Gerson, M.D.

University Services
Philadelphia, Pennsylvania

Advisors (Continued)

Lieutenant L. Dan Hoey, Ph.D.

U.S. Navy Drug Screening Lab
Great Lakes, Illinois

Michael Leher, Ph.D.

Long Island Jewish Medical Center
New Hyde Park, New York

Joerg Norbert Pirl, Ph.D., DABFT

Illinois Department of Public Health
Chicago, Illinois

R. H. Barry Sample, Ph.D.

Quest Diagnostics
Atlanta, Georgia

Michael J. Welch, Ph.D.

National Institute of Standards & Technology
Gaithersburg, Maryland

Dr. Rafael de la Torre

Institute Municipal d'Investigacio Medica
Barcelona, Spain

Jennifer K. McGeary, M.T.(ASCP), M.S.H.A.
Staff Liaison

NCCLS
Wayne, Pennsylvania

Patrice E. Polgar
Editor/Project Manager

NCCLS
Wayne, Pennsylvania

Donna M. Wilhelm
Assistant Editor

NCCLS
Wayne, Pennsylvania

Active Membership (as of 1 October 2002)

Sustaining Members

Abbott Laboratories
American Association for
Clinical Chemistry
Beckman Coulter, Inc.
BD and Company
bioMérieux, Inc.
CLMA
College of American Pathologists
GlaxoSmithKline
Nippon Becton Dickinson Co., Ltd.
Ortho-Clinical Diagnostics, Inc.
Pfizer Inc
Roche Diagnostics, Inc.

Professional Members

AISAR-Associazione Italiana per lo
Studio degli
American Academy of Family
Physicians
American Association for
Clinical Chemistry
American Association for
Respiratory Care
American Chemical Society
American Medical Technologists
American Public Health Association
American Society for Clinical
Laboratory Science
American Society of Hematology
American Society for Microbiology
American Type Culture
Collection, Inc.
Asociacion Mexicana de
Bioquímica Clínica A.C.
Assn. of Public Health Laboratories
Assoc. Micro. Clinici Italiani-
A.M.C.L.I.
British Society for Antimicrobial
Chemotherapy
CADIME-Camara De Instituciones
De Diagnostico Medico
Canadian Society for Medical
Laboratory Science—Société
Canadienne de Science de
Laboratoire Médical
Clinical Laboratory Management
Association
COLA
College of American Pathologists
College of Medical Laboratory
Technologists of Ontario

College of Physicians and
Surgeons of Saskatchewan
ESCMID
Fundación Bioquímica Argentina
International Association of Medical
Laboratory Technologists
International Council for
Standardization in Haematology
International Federation of
Clinical Chemistry
Italian Society of Clinical
Biochemistry and Clinical
Molecular Biology
Japan Society of Clinical Chemistry
Japanese Committee for Clinical
Laboratory Standards
Joint Commission on Accreditation
of Healthcare Organizations
National Academy of Clinical
Biochemistry
National Association of Testing
Authorities – Australia
National Society for
Histotechnology, Inc.
Ontario Medical Association
Quality Management Program-
Laboratory Service
RCPA Quality Assurance Programs
PTY Limited
Sociedade Brasileira de Analises
Clinicas
Sociedad Espanola de Bioquímica
Clínica y Patología Molecular
Taiwanese Committee for Clinical
Laboratory Standards (TCCLS)
Turkish Society of Microbiology

Government Members

Association of Public Health
Laboratories
Armed Forces Institute of Pathology
BC Centre for Disease Control
Centers for Disease Control and
Prevention
Centers for Medicare & Medicaid
Services/CLIA Program
Centers for Medicare & Medicaid
Services
Chinese Committee for Clinical
Laboratory Standards
Commonwealth of Pennsylvania
Bureau of Laboratories
Department of Veterans Affairs
Deutsches Institut für Normung
(DIN)

FDA Center for Devices and
Radiological Health
FDA Center for Veterinary
Medicine
FDA Division of Anti-Infective
Drug Products
Iowa State Hygienic Laboratory
Massachusetts Department of
Public Health Laboratories
National Center of Infectious
and Parasitic Diseases (Bulgaria)
National Health Laboratory Service
(South Africa)
National Institute of Standards
and Technology
New York State Department of
Health
Ohio Department of Health
Ontario Ministry of Health
Pennsylvania Dept. of Health
Saskatchewan Health-Provincial
Laboratory
Scientific Institute of Public Health;
Belgium Ministry of Social
Affairs, Public Health and the
Environment
Swedish Institute for Infectious
Disease Control
Thailand Department of Medical
Sciences

Industry Members

AB Biodisk
Abbott Laboratories
Abbott Laboratories, MediSense
Products
Acrometrix Corporation
Alifax S.P.A.
Ammirati Regulatory Consulting
Anaerobe Systems
Assessor
AstraZeneca
AstraZeneca R & D
Boston
Avant Immunotherapeutics, Inc.
Aventis
Axis-Shield POC AS
Bayer Corporation – Elkhart, IN
Bayer Corporation – Tarrytown, NY
Bayer Corporation – West Haven,
CT
Bayer Medical Ltd.
BD
BD Biosciences – San Jose, CA
BD Consumer Products

BD Diagnostic Systems
 BD Italia S.P.A.
 BD VACUTAINER Systems
 Beckman Coulter, Inc.
 Beckman Coulter, Inc. Primary Care Diagnostics
 Beckman Coulter K.K. (Japan)
 Bio-Development SRL
 Bio-Inova Life Sciences International
 Bio-Inova Life Sciences North America
 BioMedia Laboratories Sdn Bhd
 BioMérieux (NC)
 bioMérieux, Inc. (MO)
 Biometrology Consultants
 Bio-Rad Laboratories, Inc.
 Bio-Rad Laboratories, Inc. - France
 Biotest AG
 Blaine Healthcare Associates, Inc.
 Bristol-Myers Squibb Company
 Canadian External Quality Assessment Laboratory
 Capital Management Consulting, Inc.
 Carl Schaper
 Checkpoint Development Inc.
 Chiron Corporation
 ChromaVision Medical Systems, Inc.
 Chronolab Ag
 Clinical Design Group Inc.
 Clinical Laboratory Improvement Consultants
 Cognigen
 Community Medical Center (NJ)
 Control Lab (Brazil)
 Copan Diagnostics Inc.
 Cosmetic Ingredient Review
 Cubist Pharmaceuticals
 Dade Behring Inc. - Deerfield, IL
 Dade Behring Inc. - Glasgow, DE
 Dade Behring Inc. - Marburg, Germany
 Dade Behring Inc. - Sacramento, CA
 Dade Behring Inc. - San Jose, CA
 David G. Rhoads Associates, Inc.
 Diagnostics Consultancy
 Diagnostic Products Corporation
 Eiken Chemical Company, Ltd.
 Elan Pharmaceuticals
 Electa Lab s.r.l.
 Enterprise Analysis Corporation
 Essential Therapeutics, Inc.
 EXPERTech Associates, Inc.
 F. Hoffman-La Roche AG
 Fort Dodge Animal Health
 General Hospital Vienna (Austria)

Gen-Probe
 GlaxoSmithKline
 Greiner Bio-One Inc.
 Helena Laboratories
 Home Diagnostics, Inc.
 IGEN Inc.
 Immunicon Corporation
 Instrumentation Laboratory
 International Technidyne Corporation
 IntraBiotics Pharmaceuticals, Inc.
 I-STAT Corporation
 Johnson and Johnson Pharmaceutical Research and Development, L.L.C.
 Kendall Sherwood-Davis & Geck
 LAB-Interlink, Inc.
 Laboratory Specialists, Inc.
 Labtest Diagnostica S.A.
 LifeScan, Inc. (a Johnson & Johnson Company)
 Lilly Research Laboratories
 Macemon Consultants
 Medical Device Consultants, Inc.
 Merck & Company, Inc.
 Minigrip/Zip-Pak
 Molecular Diagnostics, Inc.
 mvi Sciences (MA)
 Nabi
 Nichols Institute Diagnostics (Div. of Quest Diagnostics, Inc.)
 NimbleGen Systems, Inc.
 Nissui Pharmaceutical Co., Ltd.
 Nippon Becton Dickinson Co., Ltd.
 Norfolk Associates, Inc.
 Novartis Pharmaceuticals Corporation
 Ortho-Clinical Diagnostics, Inc. (Raritan, NJ)
 Ortho-Clinical Diagnostics, Inc. (Rochester, NY)
 Oxoid Inc.
 Paratek Pharmaceuticals
 Pfizer Inc
 Pharmacia Corporation
 Philips Medical Systems
 Powers Consulting Services
 Premier Inc.
 Procter & Gamble Pharmaceuticals, Inc.
 The Product Development Group
 QSE Consulting
 Quintiles, Inc.
 Radiometer America, Inc.
 Radiometer Medical A/S
 Replidyne
 Roche Diagnostics GmbH
 Roche Diagnostics, Inc.

Roche Laboratories (Div. Hoffmann-La Roche Inc.)
 Sarstedt, Inc.
 SARL Laboratoire Carron (France)
 Schering Corporation
 Schleicher & Schuell, Inc.
 Second Opinion
 Showa Yakuhin Kako Company, Ltd.
 Streck Laboratories, Inc.
 SurroMed, Inc.
 Synermed Diagnostic Corp.
 Sysmex Corporation (Japan)
 Sysmex Corporation (Long Grove, IL)
 The Clinical Microbiology Institute
 The Toledo Hospital (OH)
 Theravance Inc.
 Transasia Engineers
 Trek Diagnostic Systems, Inc.
 Versicor, Inc.
 Vetoquinol S.A.
 Visible Genetics, Inc.
 Vysis, Inc.
 Wallac Oy
 Wyeth-Ayerst
 Xyletech Systems, Inc.
 YD Consultant
 YD Diagnostics (Seoul, Korea)

Trade Associations

AdvaMed
 Association of Medical Diagnostic Manufacturers
 Japan Association Clinical Reagents Ind. (Tokyo, Japan)
 Medical Industry Association of Australia

Associate Active Members

31st Medical Group/SGSL (APO, AE)
 67th CSH Wuerzburg, GE (NY)
 121st General Hospital (CA)
 Academisch Ziekenhuis-VUB (Belgium)
 Acadiana Medical Laboratories, LTD (LA)
 Adena Regional Medical Center (OH)
 Advocate Healthcare Lutheran General (IL)
 Akershus Central Hospital and AFA (Norway)
 Albemarle Hospital (NC)
 Allegheny General Hospital (PA)

- Allina Health System (MN)
 Alton Ochsner Medical Foundation (LA)
 Antwerp University Hospital (Belgium)
 Arkansas Department of Health
 ARUP at University Hospital (UT)
 Armed Forces Research Institute of Medical Science (APO, AP)
 Associated Regional & University Pathologists (UT)
 Aurora Consolidated Laboratories (WI)
 Azienda Ospedale Di Lecco (Italy)
 Bay Medical Center (MI)
 Baystate Medical Center (MA)
 Bbaguas Duzen Laboratories (Turkey)
 Bermuda Hospitals Board
 Bo Ali Hospital (Iran)
 Brooks Air Force Base (TX)
 Broward General Medical Center (FL)
 Cadham Provincial Laboratory
 Calgary Laboratory Services
 Carilion Consolidated Laboratory (VA)
 Cathay General Hospital (Taiwan)
 Central Peninsula General Hospital (AK)
 Central Texas Veterans Health Care System
 Centre Hospitalier Regional del la Citadelle (Belgium)
 Centro Diagnostico Italiano (Milano, Italy)
 Champlain Valley Physicians Hospital (NY)
 Chang Gung Memorial Hospital (Taiwan)
 Changi General Hospital (Singapore)
 The Charlotte Hungerford Hospital (CT)
 Children's Hospital (LA)
 Children's Hospital (NE)
 Children's Hospital & Clinics (MN)
 Children's Hospital Medical Center (Akron, OH)
 Children's Hospital of Philadelphia (PA)
 Children's Medical Center of Dallas (TX)
 Clarian Health-Methodist Hospital (IN)
 Clendo Lab (Puerto Rico)
 Clinical Laboratory Partners, LLC (CT)
 CLSI Laboratories (PA)
- Columbia Regional Hospital (MO)
 Commonwealth of Kentucky
 Community Hospital of Lancaster (PA)
 CompuNet Clinical Laboratories (OH)
 Cook County Hospital (IL)
 Cook Children's Medical Center (TX)
 Covance Central Laboratory Services (IN)
 Danish Veterinary Laboratory (Denmark)
 Danville Regional Medical Center (VA)
 Delaware Public Health Laboratory
 Department of Health & Community Services (New Brunswick, Canada)
 DesPeres Hospital (MO)
 DeTar Hospital (TX)
 Detroit Health Department (MI)
 Diagnosticos da América S/A (Brazil)
 Dr. Everett Chalmers Hospital (New Brunswick, Canada)
 Doctors Hospital (Bahamas)
 Duke University Medical Center (NC)
 Dwight David Eisenhower Army Med. Ctr. (GA)
 E.A. Conway Medical Center (LA)
 Eastern Maine Medical Center
 East Side Clinical Laboratory (RI)
 Eastern Health (Vic., Australia)
 Elyria Memorial Hospital (OH)
 Emory University Hospital (GA)
 Esoterix Center for Infectious Disease (TX)
 Fairview-University Medical Center (MN)
 Federal Medical Center (MN)
 Florida Hospital East Orlando
 Focus Technologies (CA)
 Foothills Hospital (Calgary, AB, Canada)
 Fresenius Medical Care/Spectra East (NJ)
 Fresno Community Hospital and Medical Center
 Frye Regional Medical Center (NC)
 Gambro BCT (CO)
 Geisinger Medical Center (PA)
 Grady Memorial Hospital (GA)
 Guthrie Clinic Laboratories (PA)
 Hahnemann University Hospital (PA)
 Harris Methodist Erath County (TX)
- Harris Methodist Fort Worth (TX)
 Hartford Hospital (CT)
 Headwaters Health Authority (Alberta, Canada)
 Health Network Lab (PA)
 Health Partners Laboratories (VA)
 Heartland Regional Medical Center (MO)
 Highlands Regional Medical Center (FL)
 Hoag Memorial Hospital Presbyterian (CA)
 Holmes Regional Medical Center (FL)
 Holzer Medical Center (OH)
 Hopital du Sacre-Coeur de Montreal (Montreal, Quebec, Canada)
 Hôpital Maisonneuve – Rosemont (Montreal, Canada)
 Hospital for Sick Children (Toronto, ON, Canada)
 Hospital Sousa Martins (Portugal)
 Hotel Dieu Hospital (Windsor, ON, Canada)
 Houston Medical Center (GA)
 Huddinge University Hospital (Sweden)
 Hurley Medical Center (MI)
 Indiana University
 Innova Fairfax Hospital (VA)
 Institute of Medical and Veterinary Science (Australia)
 International Health Management Associates, Inc. (IL)
 Jackson Memorial Hospital (FL)
 Jersey Shore Medical Center (NJ)
 John C. Lincoln Hospital (AZ)
 John F. Kennedy Medical Center (NJ)
 John Peter Smith Hospital (TX)
 Kadlec Medical Center (WA)
 Kaiser Permanente Medical Care (CA)
 Kaiser Permanente (MD)
 Kantonsspital (Switzerland)
 Keller Army Community Hospital (NY)
 Kenora-Rainy River Regional Laboratory Program (Ontario, Canada)
 Kern Medical Center (CA)
 Kimball Medical Center (NJ)
 King Faisal Specialist Hospital (Saudi Arabia)
 King Khalid National Guard Hospital (Saudi Arabia)
 King's Daughter Medical Center (KY)

- Klinični Center (Slovenia)
 Laboratories at Bonfils (CO)
 Laboratoire de Santé Publique du
 Quebec (Canada)
 Laboratório Fleury S/C Ltda.
 (Brazil)
 Laboratory Corporation of America
 (NJ)
 Laboratory Corporation of
 America (MO)
 LAC and USC Healthcare
 Network (CA)
 Lakeland Regional Medical Center
 (FL)
 Lancaster General Hospital (PA)
 Langley Air Force Base (VA)
 LeBonheur Children's
 Medical Center (TN)
 L'Hotel-Dieu de Quebec (Canada)
 Libero Istituto Univ. Campus
 BioMedico (Italy)
 Louisiana State University
 Medical Center
 Maccabi Medical Care and Health
 Fund (Israel)
 Malcolm Grow USAF Medical
 Center (MD)
 Martin Luther King/Drew Medical
 Center (CA)
 Massachusetts General Hospital
 (Microbiology Laboratory)
 MDS Metro Laboratory Services
 (Burnaby, BC, Canada)
 Medical College of Virginia
 Hospital
 Medicare/Medicaid Certification,
 State of North Carolina
 Memorial Hospital at Gulfport (MS)
 Memorial Medical Center (IL)
 Memorial Medical Center (LA)
 Jefferson Davis Hwy
 Memorial Medical Center (LA)
 Napoleon Avenue
 Mercy Medical Center (IA)
 Methodist Hospital (TX)
 Methodist Hospitals of Memphis
 (TN)
 MetroHealth Medical Center (OH)
 Michigan Department of
 Community Health
 Mississippi Baptist Medical Center
 Monte Tabor - Centro Italo -
 Brasileiro de Promocao (Brazil)
 Montreal Children's Hospital
 (Canada)
 Montreal General Hospital
 (Canada)
- MRL Pharmaceutical Services, Inc.
 (VA)
 Nassau County Medical Center
 (NY)
 National Institutes of Health (MD)
 Naval Hospital - Corpus Christi
 (TX)
 Naval Surface Warfare Center (IN)
 Nebraska Health System
 New Britain General Hospital (CT)
 New England Fertility Institute
 (CT)
 New Mexico VA Health Care
 System
 New York University Medical
 Center
 North Carolina State Laboratory of
 Public Health
 North Shore - Long Island Jewish
 Health System Laboratories (NY)
 North Shore University Hospital
 (NY)
 Northwestern Memorial Hospital
 (IL)
 O.L. Vrouwziekenhuis (Belgium)
 Ordre professionnel des
 technologists médicaux du
 Québec
 Ospedali Riuniti (Italy)
 The Ottawa Hospital
 (Ottawa, ON, Canada)
 Our Lady of Lourdes Hospital (NJ)
 Our Lady of the Resurrection
 Medical Center (IL)
 Pathology and Cytology
 Laboratories, Inc. (KY)
 Pathology Associates Medical
 Laboratories (WA)
 The Permanente Medical Group
 (CA)
 Piedmont Hospital (GA)
 Pikeville Methodist Hospital (KY)
 Pocono Hospital (PA)
 Presbyterian Hospital of Dallas
 (TX)
 Providence Health Care
 Queen Elizabeth Hospital (Prince
 Edward Island, Canada)
 Queensland Health Pathology
 Services (Australia)
 Quest Diagnostics Incorporated
 (CA)
 Quintiles Laboratories, Ltd. (GA)
 Regions Hospital
 Reid Hospital & Health Care
 Services (IN)
 Research Medical Center (MO)
 Rex Healthcare (NC)
- Rhode Island Department of Health
 Laboratories
 Riyadh Armed Forces Hospital
 (Saudi Arabia)
 Royal Columbian Hospital (New
 Westminster, BC, Canada)
 Sacred Heart Hospital (MD)
 Saint Mary's Regional Medical
 Center (NV)
 St. Alexius Medical Center (ND)
 St. Anthony Hospital (CO)
 St. Anthony's Hospital (FL)
 St. Barnabas Medical Center (NJ)
 St-Eustache Hospital (Quebec,
 Canada)
 St. Francis Medical Ctr. (CA)
 St. John Hospital and Medical
 Center (MI)
 St. John Regional Hospital (St.
 John, NB, Canada)
 St. Joseph Hospital (NE)
 St. Joseph's Hospital - Marshfield
 Clinic (WI)
 St. Joseph's Medical Center (NY)
 St. Joseph Mercy Hospital (MI)
 St. Jude Children's Research
 Hospital (TN)
 St. Luke's Regional Medical
 Center (IA)
 St. Mary of the Plains Hospital
 (TX)
 St. Mary's Hospital & Medical
 Center (CO)
 St. Vincent Medical Center (CA)
 Ste. Justine Hospital (Montreal, PQ,
 Canada)
 Salina Regional Health Center (KS)
 San Francisco General Hospital
 (CA)
 Santa Clara Valley Medical Center
 (CA)
 Seoul Nat'l University Hospital
 (Korea)
 Shanghai Center for the
 Clinical Laboratory (China)
 South Bend Medical Foundation
 (IN)
 Southwest Texas Methodist Hospital
 (TX)
 South Western Area Pathology
 Service (Australia)
 Southern Maine Medical Center
 Specialty Laboratories, Inc. (CA)
 Stanford Hospital and Clinics (CA)
 State of Washington Department of
 Health
 Stony Brook University Hospital
 (NY)

Stormont-Vail Regional Medical Center (KS)
 Sun Health-Boswell Hospital (AZ)
 Swedish Medical Center – Providence Campus (WA)
 Tampa General Hospital (FL)
 Temple University Hospital (PA)
 Tenet Odessa Regional Hospital (TX)
 The Toledo Hospital (OH)
 Touro Infirmary (LA)
 Trident Regional Medical Center (SC)
 Tripler Army Medical Center (HI)
 Truman Medical Center (MO)
 UCSF Medical Center (CA)
 UNC Hospitals (NC)
 University College Hospital (Galway, Ireland)
 University Hospital (Gent) (Belgium)
 University Hospitals of Cleveland (OH)
 The University Hospitals (OK)
 University of Alabama-Birmingham Hospital

University of Alberta Hospitals (Canada)
 University of Colorado Health Science Center
 University of Chicago Hospitals (IL)
 University of Illinois Medical Center
 University of the Ryukyus (Japan)
 University of Texas M.D. Anderson Cancer Center
 University of Virginia Medical Center
 University of Washington
 UZ-KUL Medical Center (Belgium)
 VA (Denver) Medical Center (CO)
 Virginia Department of Health
 VA (Hines) Medical Center
 VA (Kansas City) Medical Center (MO)
 VA (Western NY) Healthcare System
 VA (San Diego) Medical Center (CA)
 VA (Tuskegee) Medical Center (AL)

VA Outpatient Clinic (OH)
 Vejle Hospital (Denmark)
 Washington Adventist Hospital (MD)
 Washoe Medical Center Laboratory (NV)
 West Jefferson Medical Center (LA)
 West Shore Medical Center (MI)
 Wilford Hall Medical Center (TX)
 William Beaumont Army Medical Center (TX)
 William Beaumont Hospital (MI)
 William Osler Health Centre (Brampton, ON, Canada)
 Williamsburg Community Hospital (VA)
 Winn Army Community Hospital (GA)
 Winnipeg Regional Health Authority (Winnipeg, Canada)
 Wishard Memorial Hospital (IN)
 Yonsei University College of Medicine (Korea)
 York Hospital (PA)

OFFICERS

Donna M. Meyer, Ph.D.,
 President
 CHRISTUS Health

Thomas L. Hearn, Ph.D.,
 President Elect
 Centers for Disease Control and Prevention

Emil Voelkert, Ph.D.,
 Secretary
 Roche Diagnostics GmbH

Gerald A. Hoeltge, M.D.,
 Treasurer
 The Cleveland Clinic Foundation

F. Alan Andersen, Ph.D.,
 Immediate Past President
 Cosmetic Ingredient Review

John V. Bergen, Ph.D.,
 Executive Director

Susan Blonshine, RRT, RPFT,
 FAARC
 TechEd

Wayne Brinster
 BD

Kurt H. Davis, FCSMLS, CAE
 Canadian Society for Medical Laboratory Science

Lillian J. Gill, M.S.
 FDA Center for Devices and Radiological Health

Robert L. Habig, Ph.D.
 Habig Consulting Group

Carolyn D. Jones, J.D., M.P.H.
 AdvaMed

BOARD OF DIRECTORS

Tadashi Kawai, M.D., Ph.D.
 International Clinical Pathology Center

J. Stephen Kroger, M.D., MACP
 COLA

Willie E. May, Ph.D.
 National Institute of Standards and Technology

Gary L. Myers, Ph.D.
 Centers for Disease Control and Prevention

Barbara G. Painter, Ph.D.
 Pharma Micro Consultancy, LLC

Judith A. Yost, M.A., M.T.(ASCP)
 Centers for Medicare & Medicaid Services

Contents

| | |
|---|-----|
| Abstract..... | i |
| Committee Membership..... | v |
| Active Membership..... | vii |
| Foreword..... | xv |
| The Quality System Approach..... | xvi |
| 1 Introduction..... | 1 |
| 2 Scope..... | 1 |
| 3 Standard Precautions..... | 1 |
| 4 Principles of GC/MS..... | 2 |
| 4.1 Gas Chromatography..... | 2 |
| 4.2 Interface..... | 3 |
| 4.3 Mass Spectrometry..... | 3 |
| 4.4 Data Acquisition..... | 7 |
| 5 Definitions..... | 10 |
| 6 Method Validation..... | 12 |
| 6.1 Specificity..... | 12 |
| 6.2 Trueness..... | 13 |
| 6.3 Precision (Repeatability and Reproducibility)..... | 14 |
| 6.4 Linearity..... | 14 |
| 6.5 Limit of Detection..... | 15 |
| 6.6 Limit of Quantitation..... | 16 |
| 6.7 Robustness..... | 16 |
| 7 Routine Instrument and Method Performance Verification..... | 16 |
| 7.1 Gas Chromatograph..... | 16 |
| 7.2 Mass Spectrometer..... | 17 |
| 7.3 Calibration..... | 18 |
| 7.4 Quality Control/Quality Assurance..... | 19 |
| 8 Identification Principles..... | 20 |
| 8.1 Gas Chromatograph..... | 20 |
| 8.2 Mass Spectral Identification..... | 21 |
| References..... | 24 |
| Additional References..... | 26 |
| Summary of Comments and Subcommittee Responses..... | 27 |
| Related NCCLS Publications..... | 33 |

Foreword

The detection of a drug in the biological fluid of an individual can have serious professional, financial, and social consequences. It is generally accepted that detection of a drug by a screening procedure must be confirmed by a second method based on a different analytical or physical principle. The purpose of the confirmation test is to decrease the probability of false-positives and to provide additional information and assurance about the identity of the detected compound.

Gas chromatography/mass spectrometry (GC/MS) is widely accepted in both scientific and legal arenas as one of the most powerful analytical techniques for the separation, quantification, and identification of drug analytes, especially at low concentrations. Technological advances have allowed introduction of bench-top GC/MS instrumentation into forensic and clinical toxicology laboratories. Further advances will continue to move state-of-the-art techniques such as gas- and liquid-phase chemical ionization, tandem mass spectrometry, high-resolution mass spectrometry, and high-performance liquid chromatography/mass spectrometry (HPLC/MS) into routine laboratory operation. Appropriate application of these analytical tools requires that the methods used are fit for their purpose and the instruments are operating correctly.

The Division of Workplace Programs, Substance Abuse and Mental Health Services Administration of the United States Department of Health and Human Services oversees the best-known drug testing program. The U.S. National Laboratory Certification Program has issued guidance documents for laboratories involved in the federal workplace drug-testing program. A similar program is under consideration in the European Union. Confirmatory assays are also used in clinical toxicology, forensic toxicology, and athletic drug testing. Currently available guidelines are not appropriate for all drug confirmation testing. The present guideline was developed to provide assistance in developing confirmation tests that are fit for the analytical purpose in each of these areas.

This guideline addresses the instrumental and methodological issues in developing a chromatographic - mass spectrometric method, routine performance of the analysis, and continued quality assurance. The chain of custody, while an important part of any test result to be submitted to the judicial system, is not discussed here. Guidelines for sample collection and screening testing have been published. Refer to the most current edition of NCCLS document T/DM8—*Urine Drug Testing in the Clinical Laboratory* for recommendations on sample collection and screening testing.

Key Words

Athletic drug testing, clinical toxicology, drugs of abuse, forensic toxicology, gas chromatography, magnetic sector mass spectrometer, mass spectrometry, quadrupole mass spectrometer, tandem mass spectrometry

A Note on Terminology

NCCLS, as a global leader in standardization and harmonization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. NCCLS recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in NCCLS, ISO, and CEN documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. In light of this, NCCLS recognizes that harmonization of terms facilitates the global application of standards and deserves immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

Of particular note in C43-A, are several terms whereby NCCLS intends to eliminate confusion over time, through its commitment to harmonization. For instance, the term "accuracy" comprises three different concepts that ISO documents capture with three distinct terms; i.e., "accuracy," "trueness," and "bias." Also in the context of this guideline, the term "precision" is defined the way ISO defines "uncertainty." To facilitate understanding, all ISO terms are defined in the guideline's "Definitions" section under the terms "accuracy" and "precision."

All terms and definitions will be reviewed for consistency with international use, and revised appropriately during the next scheduled revision of this guideline.

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care*. The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

QSEs

| | |
|------------------------|------------------------|
| Documents & Records | Information Management |
| Organization | Occurrence Management |
| Personnel | Assessment |
| Equipment | Process Improvement |
| Purchasing & Inventory | Service & Satisfaction |
| Process Control | Facilities & Safety |

C43-A addresses the following quality system essentials (QSEs):

| Documents & Records | Organization | Personnel | Equipment | Purchasing & Inventory | Process Control | Information Management | Occurrence Management | Assessment | Process Improvement | Service & Satisfaction | Facilities & Safety |
|---------------------|--------------|-----------|-----------|------------------------|-----------------|------------------------|-----------------------|------------|---------------------|------------------------|---------------------|
| | | | | | X | | | | | | |

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*.

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information. The arrow depicts the sequence, from left to right, that any clinical laboratory follows. In addition, the necessary steps or subprocesses are listed below them.

Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Approved Guideline

1 Introduction

Gas chromatography/Mass spectrometry (GC/MS) is generally accepted as the "gold standard" for identification and quantitation of drug analytes. As such, it is frequently used to confirm presumptive positive drug screening tests performed by immunoassay, thin-layer chromatography, high-performance liquid chromatography, or gas chromatography. The confidence in the ability of GC/MS to provide unequivocal analytical data is based on recognition of its reproducibility, repeatability, specificity, and trace detection capabilities. While this confidence is well founded, the measurement and identification of trace levels of compounds in complex biological matrices such as urine, hair, blood, bile, or organ tissue present a unique problem. Since GC/MS confirmation tests are applied in areas of clinical and forensic science other than drugs of abuse testing, it seems appropriate to establish broader criteria.

2 Scope

In drug analysis, GC/MS is used either to increase confidence in the identification of an unknown compound or to improve the limits of detection or quantitation through increased analytical specificity. Because of this unique combination of identification and quantitation capabilities, GC/MS methods, particularly confirmation methods, require a specific set of criteria for validation of methods and for performance verification in routine analysis.

There are two broad classes of drug analysis performed with GC/MS instrumentation. For some compounds, quantitative concentration thresholds have been established, on scientific and administrative grounds, to determine the presence of the drug or drug metabolite. When the threshold concentration, threshold ratio of amounts, or other defined parameter is exceeded, the compound is deemed to be present or to be present in nonphysiological amounts. In these cases, the performance of the method and instrument at the threshold has particular importance. The best-known example of the threshold approach was the development of specific administrative threshold concentrations and criteria for identification of five drugs of abuse for the federal drug-testing program.¹ For some other drugs or drug metabolites, however, detection at any documentable concentration is of concern. For these nonthreshold compounds, performance criteria for identification may be more important than the ability to quantify.

Although bench top GC/MS instrumentation has become more available and easier to use, a uniform practice must be established and maintained to provide acceptable evidence in an administrative appeal hearing or legal setting. Continuing improvements in theory and instrumentation will facilitate the use of new techniques, such as GC/MS/MS, in routine analysis. Thus, there is a need to define uniform practices not only for routine GC/MS methods, but also for the application of these more sophisticated approaches.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management

of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections*.

4 Principles of GC/MS

GC/MS is one of a number of hybrid techniques that couple two analytical techniques to achieve a synergistic improvement in analytical performance. The appropriate operation of each technique is necessary to achieve an analytical performance objective.

4.1 Gas Chromatography

GC separates molecules by differences in their equilibrium distribution between a gaseous mobile phase and a liquid or solid stationary phase. The degree of separation between the different components in a mixture is affected by the mobile phase, its flow rate, the stationary phase used, and the temperature and/or rate of temperature change. The emergence of the analyte from the column gives rise to a Gaussian-shaped chromatographic peak. The time that has elapsed between injection and the time when the chromatographic peak apex appears, corresponding to the elution of the maximum concentration of analyte, is called the "retention time." If an unknown compound has the same retention time as a reference material under the same chromatographic conditions, the result is consistent with the presumption, but does not prove, that the two compounds are the same.

4.1.1 Sample Introduction

Because the analyte must be in the gas phase for separation, the sample introduction system must transform the liquid sample into a gas. There are a variety of sample introduction techniques presently available in GC, including isothermal split or splitless, temperature-programmed split or splitless, direct, and on-column. Each technique has advantages and disadvantages. All techniques require the sample entry port to be sealed to isolate the mobile phase stream from the outside environment. This requires either a septum or an alternate sealing system that is repeatedly penetrated by a needle. Because most injection techniques require sufficient heat to rapidly vaporize both the injection solvent and the analyte(s), components dislodged and volatilized from the septum can cause problems. Small amounts (ng) of analyte are introduced into the column. Due to the relatively large surface area of the injection port, its design, the inertness of the materials used, and the cleanliness of those portions which come in contact with the sample are critical for efficient, reproducible sample introduction.

4.1.2 Columns

Two types of GC columns exist: packed and open tubular (capillary). Packed columns are very infrequently used in conjunction with mass spectrometers and will not be discussed. Capillary columns are small diameter (0.10 to 0.53 mm inside diameter) tubes of fused silica. The stationary phase is a cross-linked annulus on the inner wall of the tube. The stationary phase is a polysiloxane or polysilarylene polymer backbone on which functional groups such as methyl, phenyl, cyanopropyl, or trifluoroacetyl provide sites for interaction with the compounds to be separated. The amount and polarity of the stationary phase, the temperature of the column, and the mobile phase flow rate are the major determining factors for separation of two compounds.

With respect to GC/MS, the major impact of the column, other than the ability to separate the compounds of interest, is the continuous degradation and elution of the phase, called "column bleed." Since all compounds entering the mass spectrometer contribute to the final signal, column bleed contributes to system background. Column bleed can be minimized by operating the column within the manufacturer's temperature range limits, by excluding oxygen and other contaminants from the mobile phase, by introducing samples that do not degrade the phase, and by selecting polysilarylene-based bonded phases.

4.2 Interface

The interface provides continuous introduction of the gaseous chromatographic effluent into the mass spectrometer ion source. Jet separator, membrane separator, or effusion separator interfaces were used to enrich the ratio of sample to carrier gas. Wide-bore (megabore), open-tubular GC columns generally require an "open-split" interface in which a portion of the effluent is discarded without enrichment of the sample relative to the carrier gas. With the widespread use of narrow-bore capillary GC columns, all of the column effluent can be directed into the ion source using a direct interface. In general, the interface is relatively trouble-free, although careful temperature control of the transfer line between the gas chromatograph and mass spectrometer is required.

4.3 Mass Spectrometry

The ability of mass spectrometry to obtain information related to the structure of the compound complements the separation capabilities of GC. Molecules entering the mass spectrometer are ionized and may undergo fragmentation. The pattern of fragments and their relative amounts are characteristic of the chemical structure of a compound but may not be unique for that compound. A mass spectrum is the two-dimensional plot of the relative abundance versus the mass-to-charge ratio of the ions. When the abundances of all ions in the mass spectrum are summed and plotted as a function of elution time, the plot is called a "total ion chromatogram" (TIC). An ion chromatogram is the two-dimensional plot of the abundance of a particular mass-to-charge ratio (m/z) versus the retention time of a GC/MS run.

4.3.1 Ion Source

As the GC effluent enters the ion source, either electron or chemical ionization achieves a continuous production of ions. The ion beam produced from the content of the effluent is directed to the mass analyzer by one or more electronic lenses. The lenses also provide the ions with a relatively homogenous momentum or velocity, which is important for separation of the ions in the mass analyzer.

The extent of ionization can be affected by the ionization (or filament) current, since this parameter is related to the number of electrons emitted from the filament. The temperature of the ion source may also affect fragmentation due to the kinetic nature of the ionization processes.

In the case of some ion trap designs, the ions are produced in the mass analyzer itself.

4.3.1.1 Electron Ionization

In electron ionization (EI), a beam of electrons directly bombards the GC effluent. When an electron from the filament has a near-collision with the analyte, an electron is abstracted from the molecule, resulting in the formation of an energetic cation radical. The cation radical is called the "molecular ion." The most common electron beam energy is 70 eV, since an electron with this amount of energy causes ionization of essentially all organic molecules. The cation radical can undergo a predictable and relatively reproducible fragmentation, which results in a cation and a radical. The cations formed from bond cleavage reactions are called "fragment ions." The most abundant ion is called the "base peak."

4.3.1.2 Chemical Ionization

In the chemical ionization (CI) mode, a reagent gas is introduced into a specially designed ion source. The reagent gas is bombarded and ionized by the electron beam. The reagent gas ions react with the GC effluent and chemically ionize the sample. The most common reagent gases are methane, ammonia, and isobutane. The most common types of CI reactions resulting in positive ions are proton transfers. The appearance of a protonated molecular ion and any fragmentation is a function of the gas phase proton affinity of the analyte and the reagent gas. Chemical ionization is a low-energy process and there is

usually little fragmentation in CI as compared to EI. This can decrease the amount of identification information present in the spectrum.

Negative ions can be produced either by electron capture of thermalized electrons or reaction with proton-abstrating reagents such as O^- . The former has become a method of choice for molecules containing electronegative atoms, such as the halogens contained in the benzodiazepine drug class. Negative ion chemical ionization (NICI) should be clearly indicated, since by convention CI is used for positive ions.

The ion trap presents a unique situation for CI. Because ions are stored in the ion trap, the reagent gas ions have a much longer time to react with the analyte. Thus, a much lower pressure (concentration) of reagent gas is necessary to produce an ion. This has made possible the use of more chemicals, such as acetonitrile and tetrahydrofuran, as reagent gases. Unfortunately, if ionization takes place in the ion trap, there is no opportunity to generate thermalized electrons, and thus electron-capture NICI is not possible.

4.3.2 Mass Analyzer

The mass analyzer separates the ion beam generated in the source into its component parts on the basis of their mass-to-charge ratio (m/z).

4.3.2.1 Magnetic and Electrostatic Mass Analyzers

In a magnetic analyzer, ions produced continuously in the source are accelerated toward a magnetic field by a kilovolt potential such that all of the ions have the same kinetic energy. The magnetic field, which describes a sector of a circle, separates the ion beam into its components according to their momentum. Ion beams of specific m/z are separated from each other spatially, with each beam having a unique radius of trajectory which depends on the accelerating voltage and the magnetic field radius and strength. The ion beam with a selected m/z passes through an adjustable slit and impinges on an electron multiplier where it is detected. Mass resolution can be adjusted by changing the width of the slit. Ions of different m/z can be focused on the detector by changing the field strength of the electromagnet.

An electrostatic analyzer can focus charged particles according to their kinetic energy-to-charge ratio. Since kinetic energy is related to the mass of the particle, the trajectory of an ion of particular m/z is a function of the accelerating voltage, and the radius and strength of the electrostatic field. Thus ions of specific m/z can be focused on the detector by varying the electrostatic field strength. The combination of a magnetic and an electrostatic analyzer results in a "double-focusing" instrument, which has improved mass resolution since the two analyzers use complementary ion separation principles. Detection of an ion of particular m/z can be achieved by varying either the electric or the magnetic field strength. Ion transmission and focusing can be improved by additional sectors, which can result in "triple-focusing" instruments. All commercial sector mass analyzers are double- or triple-focusing instruments.

Sector mass analyzers are important for several reasons. First, they can achieve mass resolution sufficient to determine the atomic composition of an ion. Second, because of the high accelerating voltages, almost all of the ions formed in the source are transmitted to the detector. Thus, tuning in a sector instrument involves adjusting the magnetic and electrostatic fields to transmit the correct mass, but relative ion intensities are not adjustable. Mass spectra obtained from a sector instrument are thus considered to be the "gold standard." Finally, because of the efficiency of ion transmission, a sector instrument has the highest sensitivity and lowest limits of detection of the commonly used mass analyzers.

4.3.2.2 Quadrupole Mass Spectrometer

The most popular mass analyzer is the quadrupole mass spectrometer (QMS). Ions produced continuously in the ion source are accelerated into the aperture between two pairs of parallel rods. Direct-current (DC)

and radio frequency (RF) voltages on opposite pairs of rods deflect the ion in a plane perpendicular to its movement down the long axis of the rods. The DC voltage, RF voltage and frequency, and the geometry of the rods determine the m/z ratio of the ion whose stable trajectory allows passage through the filter. Ions of other m/z either collide with the rods or are pumped away by the vacuum system. A selected m/z ion can be transmitted through the rods by selecting the appropriate DC and RF voltages. By simultaneously increasing the DC and RF voltages in a fixed ratio, ions of increasing m/z are sequentially transmitted to the detector.

The voltages applied to accelerate the ions from the source must be low (e.g., a few volts) so that the ions spend sufficient time in the quadrupole field to obtain good mass resolution. This can result in decreased transmission efficiency for high mass ions. In addition, the absolute DC and RF voltages applied and their ratio during the scan influence the transmission efficiency and apparent width of the mass peak detected. Thus, in tuning a QMS in the scan mode, the trueness of the mass axis, the relative intensities of the ions, and the width of the mass peak (and thus mass resolution) can be adjusted. This has significant implications for mass spectral library searching and matching.

Transmission of a specific ion is a different process. Because the transmission of ions with other m/z is not of concern, DC and RF voltages are chosen which generate the largest chromatographic peak profile, usually by selecting a specific mass (e.g., 432.3). In addition, the DC/RF ratio can be selected to transmit more ions at the cost of decreased mass resolution. This will increase signal and may increase signal-to-noise ratio if no ions of similar m/z are in the background or matrix. It may be appropriate in this operating mode to select targeted tuning conditions that will not result in a spectral scan with accepted relative ion intensities for a reference compound like perfluorotributylamine (PFTBA).

4.3.2.3 Ion Trap

The ion trap also separates ions through their interaction with a quadrupole field generated by RF and DC voltages. The ion trap can operate in several modes: selected mass detection, selected mass storage, and selected mass ejection. In the selected mass ejection mode used on most commercially available ion traps, all ions formed during ionization are stored within a space surrounded by the ring and end-cap electrodes by application of an RF voltage to the ring electrode. A linear increase in RF voltage causes instability in the trajectory of ions of increasing m/z which results in their ejection from the ion trap. The ions are detected with an electron multiplier located outside of the ion trap. A variety of other electronic wave functions can be applied to the end-cap electrodes of the ion trap in order to improve performance or facilitate ion reactions such as mass spectrometry/mass spectrometry.

4.3.2.4 Time-of-Flight Mass Spectrometers

Time-of-flight (TOF) mass spectrometers separate ions based on the time required for the ions to travel a defined distance after acceleration in an electrical field. Higher-mass ions have lower velocities than lower-mass ions. One advantage of TOF MS is the extremely rapid scan speed that can be used in conjunction with "fast GC." The TOF analyzer is also capable of higher-mass resolution than the QMS, depending on the speed and handling of data acquisition. Due to the nature of the data acquisition, TOF MS instruments always operate in the full-scan mode.

4.3.3 Mass Spectrometry/Mass Spectrometry

Mass spectrometry/mass spectrometry (MS/MS) has been a relatively recent addition to analytical methodology. In this approach, a molecule is ionized and the molecular ion or a fragment ion is separated from other ions in a mass analyzer as described above. This precursor ion (also known as a "parent ion") is then focused into a collision cell where it undergoes an energetic collision with a target (or collision) gas. The collision energy is partially transformed into potential energy, which results in fragmentation to form product ions (also known as "daughter ions"). This process is called "collision-induced dissociation"

(CID). The product ions are then analyzed and either a spectrum of product ion mass abundances or a selected reaction product can be monitored. The main experimental parameters that affect the CID process are the collision energy, determined by voltages within the instrument, and the collision gas thickness (or pressure).

There are two fundamentally different approaches to MS/MS employing either kilovolt collision energies (magnetic/electrostatic mass analyzers) or volt collision energies (quadrupole and ion trap mass analyzers). Because of the energy differences, the spectra obtained are frequently different. High-energy CID applications often use H_2 as the collision gas. The collision cell is usually an open design, since the velocity of the precursor ion and the low mass of the collision gas result in relatively little scattering of the product ions. In contrast, most low-energy CID applications use argon (He in ion traps) as the collision gas, and must use a collision cell that refocuses the product ion beam. In the QMS, the collision cell is usually a set of RF-only quadrupole rods into which the collision gas flows. This approach is sometimes called "MS/MS in space." In the ion trap, the CID process occurs between the stored precursor ion and the helium bath gas. The collision energy is provided by application of a voltage waveform to the end cap electrodes. Product ions are stored with the same RF field, and ejected using a mass instability scan. Since the product ions are not separated spatially from the precursor ions, this technique is sometimes referred to as "MS/MS in time." In the ion trap, the collision gas thickness cannot be varied, but the time for reaction between the precursor and the collision gas can be controlled to vary the CID product ions.

Although MS/MS spectra can be obtained, there are no spectral libraries and the rational explanation of the fragmentation processes is not as well developed as for electron ionization MS. Thus, the main analytical application of GC/MS/MS to toxicology is selected reaction monitoring (SRM), where several product ions are selectively monitored as a function of time. Comparison of several product ion abundances between a reference standard and an unknown under the same GC/MS/MS conditions should allow the development of identification criteria.

It should be noted that introduction of another collision process and mass analysis could give rise to an MS/MS/MS spectrum or third-generation product ion for quantitation. It is also noteworthy that sector instruments are capable of monitoring metastable ions. In this approach, unstable ions produced in the ion source can fragment in the field free region between the ion source and the magnetic or electrostatic mass analyzer. Since the resulting metastable ion has the momentum of its precursor but the mass of the fragment, it gives a unique "apparent" mass. Monitoring of metastable ions can provide selectivity similar to that obtained from MS/MS. Because quadrupole and ion trap analyzers do not use the same mass separation principle, metastable ions are not observed.

4.3.4 Vacuum System

The dependence of the mass separation on the specific trajectory of an ion through the mass analyzer means that the ion cannot collide with any other molecules along its path. The observed pressure in a container is the result of molecules colliding with the walls of the container or each other. Therefore mass separation devices must be operated at a sufficiently low pressure that the path the ion takes from the source to the detector is shorter than the distance between collisions with other molecules. The relationship between pressure and the mean-free path is well established. The longer the path through the analyzer, the lower the operating vacuum requirements.

There are two exceptions to the requirement for high vacuum in a mass analyzer. It has been clearly demonstrated that the presence of a low-molecular-weight gas, such as helium, in the ion trap resulted in improved mass resolution. This is the result of the helium bath gas decreasing the energy dispersion of the ions in the trap, and moving them toward the center of the ion trap where they were more efficiently ejected. A similar finding of improved transmission efficiency and mass resolution was recently demonstrated for higher pressures in a collision cell for low-energy MS/MS.

The presence of large amounts of air from a leak can influence ionization efficiency, mass resolution, and system sensitivity. It can also cause electrical arcing and system failure, particularly in systems in which high voltages are used. In smaller amounts, it can increase the need for routine maintenance due to detrimental reactions with the filament, column, or other components of the system.

4.4 Data Acquisition

The use of a mass analyzer provides the ability to acquire and analyze data in a variety of ways (see Figure 1). In the scan mode, the components of the total ion beam having a specific m/z are focused sequentially on the detector. The width of the mass peak focused on the detector depends on the mass analyzer used and the computer control of the electronic signal processing. For quadrupole analyzers, from six to ten collections are made across a one-mass unit window. For double-focusing instruments, the ion optics and the width of the collection slit determine the mass resolution. Unless scan times must be minimized, the spectrum should be collected from above the ions observed from air (m/z 35 to 50), to well above the expected molecular weight of the sample. In the case of CI, the scan should begin above the m/z of the highest mass reagent gas ions. The resulting plot of relative ion abundance as a function of the mass-to-charge ratio is the mass spectrum. The ion with the highest abundance in a mass spectrum is termed the "base peak" and is normalized to 100%. It is common practice to report other ion fragment abundances as percentages of the base peak height. The molecular ion (M^+), that results from detection of the radical cation formed during EI, corresponds to the molecular weight of the compound. Observation of the molecular ion is an important contributor in the identification of a compound. In cases where the molecular ion is not observed, CI can be used to determine the molecular mass.

The application of the scan mode to drug analysis is frequently limited by the fact that the target compounds are present at a concentration too low to provide a reliable mass spectrum. For magnetic and electrostatic analyzers and the quadrupole mass spectrometer (QMS), selecting a specific m/z of interest and monitoring it for defined periods of time can enhance the signal-to-noise ratio. This approach is called "selected ion monitoring" (SIM) for single-mass analyzers and "selected reaction monitoring" (SRM) for tandem-mass analyzers.

The following summarizes current thinking regarding the use of the spectral scan versus SIM mode of operation.²

- Qualitative analysis: Whether it is better to scan the spectral mass spectrometer repetitively over the full mass range, or to monitor only selected ions, continues to be debated. Both modes of operation have their strengths and limitations, so it is unwise to adhere rigidly to only one mode of operation. A good quality full-scan mass spectrum generally provides the best qualitative identification; however, the SIM mode generally is more sensitive and less affected by potential interferences from coeluting compounds. The specificity (certainty of identification) of a SIM assay depends on many factors including: the number of ions monitored; the uniqueness of the monitored ions; the selectivity of the extraction procedure; the type of derivative; the efficiency of the chromatographic separation; and the selectivity of the method of ionization. A well-designed SIM assay can provide a very reliable method of identification. However, it may be difficult to evaluate the reliability of a SIM assay without personal experience with the method or access to data from the analysis of a substantial number of specimens. With either mode of mass analysis, the analyte's retention time (or better, its retention time relative to a reference standard) should agree well with the analyte's expected (relative) retention time.
- Quantitative analysis: The accuracy of quantitative measurements performed by GC/MS is highly dependent on the intensity of the analyte's ion current relative to the background ion current ('noise') intensity. Acceptable quantitative measurements can be obtained from reconstructed ion chromatograms, or total ion current chromatograms obtained under full-scan data acquisition, if a

relatively high concentration of analyte is present in the specimen. However, when analyte concentrations are in the low-nanogram/milliliter range, it is generally necessary to use selected ion monitoring to obtain sufficient ion current intensity for accurate quantitation.

For the purposes and definitions of this guideline, the quantitative analysis comments relate to the analysis of threshold compounds and some nonthreshold compounds, while the qualitative analysis comments relate exclusively to the analysis of nonthreshold compounds.

The ability to confirm the identity of compounds based on the relative intensity of SIM ions was originally investigated by Sphon.³ Using the spectrum of diethylstilbesterol as a model, a unique identification in a library of 30,000 compounds was obtained by comparing three ions from the spectrum at unit mass resolution with $\pm 30\%$ agreement between relative intensities of the base peak and $\pm 35\%$ agreement between relative intensities of the other two ions. Sphon concluded that three ions are regarded as a minimum, depending on the specificity of the ions monitored. It is important to note that the compound was not derivatized and that chromatographic resolving power of capillary GC was not available. More recent evaluation of these criteria against a larger database has confirmed the validity of the process.⁴

The fundamental basis of identification of a compound is the information content of the analytical data. Using this approach, other investigators have attempted to determine "selectivity indices" based on the inherent specificity of each step in the analytical procedure.⁵ The information content of GC/MS/MS has been investigated by Fetterolf and Yost.⁶

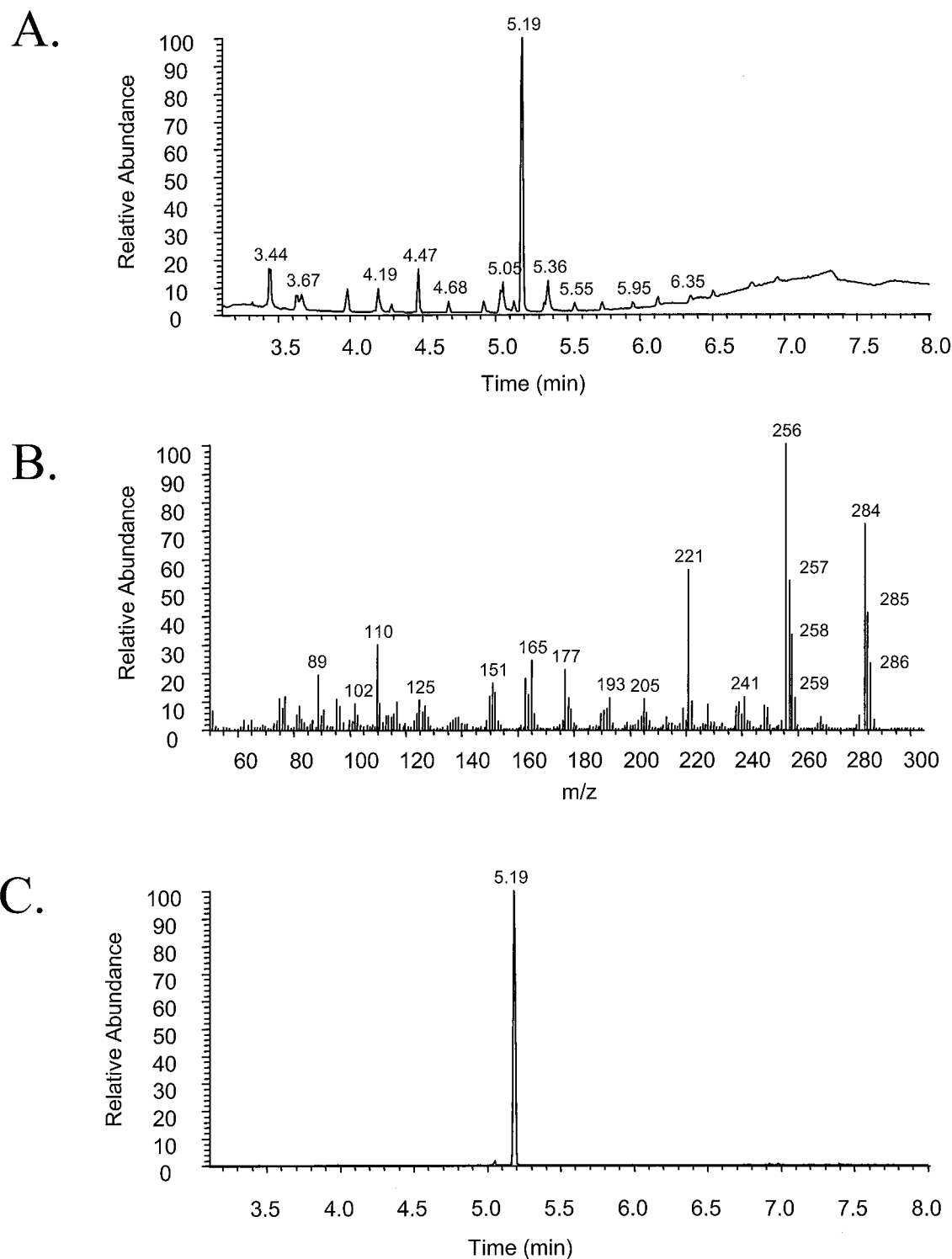


Figure 1. A) Representative GC/MS trace shown as a total ion chromatogram. B) Full scan mass spectra of diazepam, the peak that eluted at 5.19 minutes. C) Selected ion chromatogram (284 m/z) of the same data shown above.

5 Definitions^a

Accuracy//Measurement accuracy//Accuracy of measurement - 1) Closeness of the agreement between the result of a measurement and the accepted reference value of the measurand [analyte] **NOTE:** In the context of this guideline, accuracy comprises the following three concepts, described by the following three distinct ISO 3534-1 terms: **Accuracy** - Closeness of the agreement between a test result and the accepted reference value; **Trueness** - Closeness of agreement between the average value obtained from a large series of test results and an accepted reference value; and **Bias** - The difference between the expectation of the test results and an accepted reference value; **NOTE:** In general, the deviation/difference is based on replicate measurement using an accepted (definitive, reference, or designated comparison) method and the method being tested, and expressed in the units of the measurement or as a percentage.

Bias - See Accuracy.

Best measurement capability - The smallest uncertainty of measurement a laboratory can achieve for a stated calibration under specified laboratory conditions.⁷⁻¹⁰

Chemical ionization, CI - The formation of new ionized species when gaseous molecules interact with ions; **NOTE:** The process may involve transfer of an electron, a proton, or other charged species between the reactants. When positive ion results from chemical ionization (CI), the term may be used without qualification; when a negative ion results, the term “negative ion chemical ionization” can be substituted. Specifics relating to ionization should be given, e.g., if negative ions are formed from sample molecules via resonance capture of thermal electrons generated in a CI source, this should be specified.¹¹

Collision-induced dissociation, CID - An ion/neutral process wherein the (fast) projectile ion is dissociated as a result of interaction with a target neutral species; **NOTE:** This is brought about by conversion during the collision of part of the translational energy of the ion to internal energy in the ion.¹¹

Drug - Any substance which when absorbed into a living organism may modify one or more of its functions.¹²

Drug of abuse - Drug used for a nontherapeutic purpose.¹²

Electron ionization, EI - Ionization of any species by electrons; **NOTE:** Electrons and photons do not “impact” molecules or atoms. They interact with them in ways that result in various electronic excitations including ionization. For that reason it is recommended that the terms “electron impact” and “photon impact” be avoided.¹¹

Electrostatic analyzer - A velocity-focusing device for producing an electrostatic field perpendicular to the direction of ion travel (usually used in combination with a magnetic analyzer for mass analysis); **NOTE:** The effect is to bring to a common focus all ions of a given kinetic energy.¹¹

Extracted ion chromatogram, EIC - Describes the processing of data from a mass spectrometer in which the ion current at one (or several) m/z values acquired in the spectral scan mode are selected and displayed as a function of time.¹¹

Ion current - The intensity of an ion beam produced in the source, passed through the mass analyzer, and measured by the detector.¹¹

^a Some of these definitions are found in NCCLS document NRSL8—*Terminology and Definitions for Use in NCCLS Documents*. For complete definitions and detailed source information, please refer to the most current edition of that document.

Ion ratio - The ratio of signal intensities at two m/z values, usually expressed as a percentage; **NOTE:** The ratio may be determined from the ratio of chromatographic peak areas or peak heights, or may be calculated from a single mass spectrum.¹¹

Limit of detection, LOD - 1) The smallest amount or concentration of analyte that can be distinguished from background at a stated confidence level; **NOTE:** For GC/MS or GC/MS/MS confirmation analysis, the compound must also satisfy identification criteria in order to be deemed detected; **2)** the lowest amount of analyte in a sample which can be detected but not quantified as an exact value.⁷⁻¹⁰

Limit of quantitation, LOQ - The lowest amount of analyte in a sample that can be quantitatively determined with {stated} acceptable precision and {stated, acceptable} bias under stated experimental conditions.⁷⁻¹⁰

Magnetic analyzer - A direction-focusing device that produces a magnetic field perpendicular to the direction of ion travel; **NOTE:** The effect is to bring to a common focus all ions of a given momentum with the same mass-to-charge ratio.¹¹

Mass spectrum - A spectrum obtained when ions (usually in a beam) are separated according to the mass-to-charge ratios of the ionic species present; **NOTE:** This plot is a graphical representation of m/z versus the measured abundance information.¹¹

m/z - An abbreviation used to denote the dimensionless quantity formed by dividing the mass of an ion by the number of charges carried by the ion; **NOTE:** It has long been called the “mass-to-charge ratio” although m is not the ionic mass nor is z a multiple of the electronic charge, e^- .¹¹

Nonthreshold substance - A compound for which detection and identification of any amount of compound is considered to be a “positive” or “present.”

Peak resolution, R_s - The separation of two peaks ($t_{R2} > t_{R1}$) in terms of their average peak width at base (w_b): $R_s = 2(t_{R2} - t_{R1}) / (w_{b1} + w_{b2})$; **NOTE:** In the case of two adjacent peaks it may be assumed that $w_{b1} = w_{b2}$, and thus, the width of the second peak may be substituted for the average value: $R_s = (t_{R1} - t_{R2}) / w_{b2}$.¹³

Plate number - A number indicative of column performance, calculated from the following equations which depend on the selection of the peak width expression:

$$N = 5.545 (V_R / w_b)^2 = 5.545 (t_R / w_b)^2$$

In these expressions the units for the quantities inside the brackets must be consistent so that their ratio is dimensionless: i.e., if the numerator is a volume, then peak width must also be expressed in terms of volume.¹³

Precision - In the context of this guideline, precision is defined the way ISO 3534-1 defines **Uncertainty** (defined below).

Precursor ion - An electrically charged molecular moiety which may dissociate to form fragments, of which one or more may be electrically charged, and one or more are neutral species; **NOTE:** A precursor ion may be a molecular ion or an electrically charged fragment of a molecular ion.¹¹

Product ion - An electrically charged product of a reaction of a particular precursor or parent ion; **NOTE:** In general, such ions have a direct relationship to a precursor ion and indeed may relate to a unique state of the precursor ion.¹¹

Radical ion - An ion containing an unpaired electron that is thus both an ion and a free radical; **NOTE:** The presence of the odd electron is denoted by placing a dot alongside the symbol for the charge.¹¹

Selected ion monitoring, SIM - Describes the operation of a mass spectrometer in which the ion currents at one (or several) selected m/z values are recorded rather than the entire mass spectrum; **NOTE:** The use of terms "multiple ion detection (MID)," "multiple ion (peak) monitoring (MPM)," and "mass fragmentography" are not recommended.¹¹

Selected reaction monitoring, SRM - Describes the operation of a tandem mass spectrometer in which the product ion currents at one (or several) selected m/z values are recorded rather than the entire mass spectrum.¹¹

Sensitivity (analytical) - Change in the response of a measuring system or instrument divided by the corresponding change in the stimulus (e.g., analyte concentration).^{7,8,9,10}

Analytical specificity - In Quantitative Testing, the ability of an analytical method to determine only the component it purports to measure or the extent to which the assay responds only to all subsets of a specified analyte and not to other substances present in the sample.

Tandem mass spectrometry, MS/MS - A technique in which an ion at a particular m/z value is isolated in one mass analysis procedure, caused to undergo fragmentation, and the products of the fragmentation process analyzed in a second mass analysis procedure; **NOTE:** The use of the abbreviation "TMS" is discouraged.¹¹

Threshold substance - A compound for which a concentration has been specified, on either an administrative or scientific basis, above which the compound is deemed to be "positive" or "present" and below which the compound is deemed to be "negative" or "not detected."

Total ion chromatogram, TIC - The sum of all the separate ion currents carried by the different ions contributing to the mass spectrum plotted as a function of time.¹¹

Trueness - See Accuracy.

Uncertainty - An estimate attached to a test result which characterizes the range of values within which the true value is asserted to lie.

6 Method Validation

All methods must be validated in a manner appropriate for the final use of the data generated by the method. A GC/MS confirmation method need not be specific for a single compound. When multiple drugs and metabolites are confirmed in the same method, validation data must be available for each compound. For threshold compounds and nonthreshold compounds for which quantitative data is determined, validation of the procedure should include all of the following parameters. Nonthreshold substances for which only detection and/or identification are reported should have specificity, limit of detection, and robustness determined, when possible.

Validation studies should be repeated if any change is made to the procedure that could affect the results.

6.1 Specificity

An investigation of specificity should be conducted during the validation of the assay. The confirmation test should be able to discriminate between compounds of closely related structures that are likely to be present. The specificity of an assay can be established by obtaining negative results in a suitable number

of known negative samples, combined with positive results in either known positive samples or negative samples spiked with a traceable reference material. The specificity of the assay may also be determined by comparison of results to those obtained by a well-characterized reference method.

In addition, the assay should be performed with compounds structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of potentially interfering materials should be based on reasonable scientific judgment with a consideration of the interferences that could occur. Elimination of a compound from consideration as an interference may also be based on scientific judgment and the structure of the potential interference.

It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte. For identification of nonthreshold substances, where the screening procedure might have similar characteristics, this may be an important consideration. In this case, a combination of two or more analytical procedures (different extraction, different chromatographic conditions, different derivative, etc.) is recommended to achieve the necessary level of specificity.

6.1.1 Specific Recommendations for Nonthreshold Compounds

In circumstances where a traceable reference standard is not available (e.g., a metabolite of a drug), the use of either a biological fluid obtained after a documented ingestion of the drug of interest or a metabolite isolated from a biological sample is acceptable as a secondary reference material. The compound should be characterized to verify its identity. Comparison of GC/MS data obtained from analysis of the biological fluid or isolate in the laboratory to mass spectra published in a reputable scientific journal is acceptable verification.

The secondary reference material should be analyzed in the same analytical run as the unknown.

6.2 Trueness

Trueness is the ability of the analytical procedure to measure the true concentration or amount of analyte. The measure of trueness is called bias. There are several methods of determining bias: repeated measurement of an analyte of known purity (e.g., a reference compound); or comparison of the measured results of the proposed analytical procedure to those of a second, well-characterized procedure whose trueness is stated or defined. Comparison of measured results of the proposed analytical procedure to those obtained as a mean of a proficiency testing survey can be used to measure bias but should be used with caution because proficiency testing results are subject to non-specific and unpredictable matrix interference. Data for determining the bias of a procedure may be obtained concurrently with precision, linearity, and specificity data.

Bias should be assessed over the entire range of expected concentrations. A mean of at least three determinations at each concentration should be used to assess bias. The bias of the method should be determined for at least three concentrations. It is recommended that one of the concentrations be near the threshold concentration, where appropriate.

These measures of trueness should be evaluated at least every twelve months or when major maintenance is performed.

6.2.1 Carryover

Contamination of a sample with residual drug from another sample or standard seriously degrades the accuracy of the analysis. Procedures should be developed and instrumental conditions should be selected to minimize or eliminate carryover. Carryover can originate from the injection syringe, wash reservoir solvents, injection port, or column. The extent of carryover should be determined under specified

conditions. In the event of potential carryover during an analytical run, the procedure should contain specific instructions for eliminating carryover and obtaining an accurate result. An example of such a procedure would be inclusion of a blank injection prior to injection of each sample of interest.

6.3 Precision (Repeatability and Reproducibility)

Repeatability is the ability of the method to provide closely similar results for the same measurand under the same conditions of measurement in a short time frame. Repeatability is sometimes called within-run imprecision. Reproducibility is a measure of imprecision when the conditions of measurement vary across time, technicians, or laboratory equipment. There are within-lab and between-lab measures of reproducibility. Within-lab reproducibility can also be called between-run reproducibility. Reproducibility between laboratories is frequently assessed by means of an interlaboratory trial or by measurements of a control material over time.

6.3.1 Specific Recommendations for Threshold Compounds

Repeatability (within-run precision) should be assessed at a minimum of three concentrations covering the specified range for the procedure using a minimum of three replicates at each concentration.

Reproducibility should be estimated when multiple instruments and analysts are used in a procedure.

Both within-run and between-run precision can be determined simultaneously using appropriately designed experiments with multiple determinations on each of several days. Appropriate analysis of variance experiments can identify the relative contribution of several sources of variability in a method.

These measures of precision should be evaluated at least every twelve months or when major maintenance is performed.

6.4 Linearity

Linearity should be established by visual evaluation of a plot of peak height or area (or in the case of an internal standard method, peak height ratio or area ratio) as a function of analyte concentration. For establishing linearity, a minimum of five concentrations is required. Linearity should be established across a range of concentrations that reflects those expected in the biological matrix.

If there is a linear relationship, test results should be evaluated by calculation of a regression line by the method of least squares. Data from the regression line may also be useful to provide mathematical estimates of the degree of linearity. The residuals from the regression line as a function of concentration should be evaluated for a random distribution to support a linear relationship. The correlation coefficient can have serious limitations, particularly if the concentration range investigated is greater than one order of magnitude.¹⁴ Despite this limitation, the correlation coefficient is a useful indicator of linearity if it is greater than 0.998.¹⁵ An alternative to this is to plot the response factor (peak area or height divided by concentration) as a function of concentration, which should be a constant. A more robust and technically difficult method has been described.¹⁶

The linear range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, bias, and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The linear range should be re-evaluated every twelve months or when major maintenance has been performed.

The standard concentrations for the calibration curve should be selected to minimize the uncertainty of the linear regression line near the threshold concentration. The best precision (lowest uncertainty) about

the regression line occurs at the mean of the standard concentrations. Where practical, the standard concentrations for the analysis should be selected so that their mean lies near the threshold concentration.

For more information on evaluating an instrument or quantitative analytical method on the basis of the manufacturer's linearity claim, see the most current version of NCCLS document EP6—*Evaluation of the Linearity of Quantitative Analytical Methods*.

6.5 Limit of Detection

A variety of methods have been proposed to determine the limit of detection (LOD) or best measurement capability.¹⁷⁻²⁴ Most of these methods rely on signal strength from a single channel of analytical data and the ability to distinguish a difference between the signal and the background at some level of confidence.¹⁷ The LOD depends strongly on the matrix background "noise" as well as instrumental noise. In determining the LOD, the method should consider the probability of false-positive and false-negative results as well as true positive and negative results.

GC/MS provides multichannel detection capability by virtue of the simultaneous detection of multiple m/z ratio ions as a function of retention time. As a result, GC/MS data contain both identification and quantification information. For the purposes of this guideline on confirmation, detection requires both a signal discernable from the background and satisfaction of identification criteria used for the method.

Historically, two methods have been used to determine the LOD in toxicology: the statistical approach and the empirical approach.²⁴ The statistical approach requires analysis of ten replicates of a known negative sample, measurement of the system noise, and calculation of a concentration corresponding to three times the matrix noise level. The empirical approach requires analysis of a series of decreasing concentrations of drug in the biological matrix. The LOD is the concentration at which it is no longer possible to detect and identify the drug.

Other methods have included the minimum detectable limit,²² and probability of detection.²³ For qualitative methods, information theory has been applied to estimate a probability of identification.^{25,26} In general, methods to determine the probability of identification require more extensive studies because nonparametric statistical approaches are used.

The LOD should be verified every twelve months or when major maintenance is performed.

6.5.1 Recommendations for Threshold Compounds

The empirical method is recommended for determining the LOD. Serial dilutions of a sample with a known concentration should be made using an appropriate biological matrix as diluent. Given the role of the matrix "noise" in detection, more than one biological matrix diluent may be desirable. The LOD is the lowest concentration where both detection and identification criteria are met. The LOD should be verified by triplicate analysis of a sample where all replicates meet criteria for detection and identification. Any bias between the concentration measured and that expected should not be considered.

6.5.2 Specific Recommendations for Nonthreshold Compounds

The empirical method may be applied to estimate the LOD for nonthreshold compounds that are not quantified. In the case where no reference standard is available, a sample for which the concentration has been estimated may be used. The procedure should clearly state the conditions under which the LOD was determined.

6.6 Limit of Quantitation

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be quantitatively detected with a stated acceptable uncertainty and bias under stated experimental conditions. For GC/MS confirmation analyses, the concentration measured in the appropriate matrix to which a suitable reference material has been added should be within $\pm 20\%$ of the expected value (bias) with a coefficient of variation not greater than 20%. The imprecision of the analysis should be determined using a minimum of three measurements.

The LOQ should be verified every twelve months or when major maintenance is performed.

6.7 Robustness

Robustness is the reliability of an analysis with respect to variations in method parameters. If the results are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. A set of system suitability parameters (e.g., resolution test, minimum signal or signal-to-noise ratio) should be developed to ensure that the validity of the analytical procedure is maintained whenever used (see Section 7).

Typical GC/MS procedure parameters to be evaluated in determining robustness may include:

- stability of analytical solutions;
- hydrolysis conditions (e.g., solutions, temperatures);
- different lots of extraction materials;
- derivatization conditions (e.g., solutions, temperatures);
- stability of analytes after derivatization;
- injection parameters (e.g., volume, temperature, flows);
- different instruments;
- different columns (different lots and/or suppliers);
- temperature (isothermal or programmed) variation;
- flow rate variation; and
- different analysts.

7 Routine Instrument and Method Performance Verification

A GC/MS confirmation method should contain, or refer to, a procedure for documenting proper performance of the analytical instrumentation prior to analyzing any samples. In addition to verifying instrument function daily, quality control samples should be included with each batch of samples to ensure method performance. The selection of the quality control sample should reflect the intended purpose of the analysis.

7.1 Gas Chromatograph

Capillary column gas chromatography is used for the majority of separations used for GC/MS confirmation work. The primary criteria for suitable chromatographic performance are retention time reproducibility, peak narrowness and asymmetry, peak resolution, and signal-to-noise ratio (or signal) at a specified concentration. It is important to note that the peak criteria reflect injector, column, and detector performance at the time of the confirmation. The compounds selected for use in evaluation of the system should be suitable for that purpose. The chromatographic system performance verification mixture should be run at least once each day and must be run after a system repair or modification prior to analysis of any specimen.

Prior to analyzing samples, the chromatographic system should be evaluated for adequate performance. Peak narrowness and asymmetry are good measures of the efficiency of the chromatographic system. The performance verification mixture should include an appropriate compound for which these parameters are monitored. Peak narrowness should be measured by the plate number measured at the half-height of the peak profile. Minimum acceptable values for these criteria must be included in the quality assurance portion of the procedure.

Peak asymmetry is generally measured as the ratio of the distance from a perpendicular dropped from the peak apex to the peak profile at a point 10% of the distance from the baseline to the peak apex. A perfectly symmetrical peak has an asymmetry value of 1.0. A peak for which the distance to the trailing edge is longer than that to the leading edge has a value greater than one and is called "tailed." A peak for which the distance to the leading edge is longer than that to the trailing edge has a value less than one and is called "fronted." The procedure must define an acceptable range of values for peak asymmetry.

A pair of appropriate compounds should be included in the performance verification mixture that assesses the ability of the system to clearly separate the compounds. The test compounds should represent the type of compounds that are being separated in the analysis. The procedure should define an acceptable range of values for resolution. The resolution may be expressed either as the defined calculation for resolution (R_s) or as the depth of valley between the peak apices. For the purposes of this discussion, the valley point height should be measured as a function of the height of the smaller peak. The minimum resolution between an adjacent pair of peaks should be at least 1.25 or approximately 10% valley/peak ratio. The rationale for recommending this chromatographic resolution is that it has been shown that peak height measurements maintain a deviation of less than 1% from the true height (accuracy) at this resolution over a 100-fold ratio of peak sizes. A similar accuracy in peak area can be obtained over a 32-fold range.²⁷

System response to a known concentration should also be documented. One or more compounds in the system performance mixture should be evaluated for either absolute signal or signal-to-noise ratio at a specified concentration. The internal standard may also be used for this assessment of system function.

7.2 Mass Spectrometer

7.2.1 Mass Axis and Abundance Calibration

A reference compound, such as perfluorotributylamine (PFTBA), decafluorotriphenylphosphine (DFTPP), or other appropriate compound, must be used to calibrate the mass axis of the mass analyzer. In the case of QMF and ion trap instruments, the reference compound is also used to set the relative abundance of selected ions as part of the tuning process.

7.2.1.1 Acceptance Criteria for the Tuning Compound Perfluorotributylamine

The laboratory must establish acceptable ranges for the relative abundance of ions m/z 69, m/z 219, and m/z 502. Widely accepted specifications for EI QMF instruments are: m/z 69, 70 to 100% of the base peak; m/z 219, greater than 20% of the base peak; and m/z 502, greater than 2% of the base peak. These specifications were developed primarily to minimize the effect of tuning on computerized library spectral matching algorithms. Manufacturers' recommendations should be considered in establishing the relative abundance ranges. Acceptance criteria should also include inspection of the mass peaks for symmetry and the absence of precursors. There should also be resolution between each major ion and its ^{13}C -isotope mass peak. In addition, it is important to monitor the consistency of the ion abundance and instrument settings between days. If an unacceptable change is observed, the underlying reason for such a change should be determined, and the problem corrected. All corrective actions must be documented.

Target tuning for high-mass compounds or selected ion monitoring (SIM) operation is acceptable. Although this approach to tuning precludes interlaboratory comparisons of resulting spectral data, the

increase in sensitivity is usually advantageous. Methodological considerations may require that the m/z 502 ion relative abundance be as high as 50%.

When tuning tandem mass spectrometry instrumentation, the manufacturer's recommendations should be considered.

7.2.1.2 Acceptance Criteria for Alternative Tuning Compounds

Alternative tuning compounds may be used for specific purposes, such as performance enhancements for measurements associated with high molecular weight compounds, for negative ion detection, or for advanced modes of operation such as tandem mass spectrometry (MS/MS). The laboratory must establish acceptable ranges for the relative abundance of ions based on criteria in the peer-reviewed literature or by the instrument manufacturer.

7.2.2 Air Leak Criteria

Proper operation of the mass spectrometer also requires that minimal amounts of air (and water) are contained in the system. The presence of large amounts of air from a leak can influence ionization efficiency, mass resolution, and system sensitivity. The laboratory must establish acceptance criteria for the presence of these compounds.

The recommended approach is to monitor the ions produced from the constituents of air itself. In a leak-free system which has been under vacuum for some time, nearly all the components of air will be pumped away, but water will be observed at m/z 17 and 18 due to the difference in the pumping efficiency for water. If the abundance ratio of m/z 28 (N_2^+) to m/z 18 (H_2O^+) is less than 0.5, the system is free of leaks. If the ratio of m/z 28 to m/z 18 is greater than 2 to 1, there is an air leak. In the case of a leak, the ratio of m/z 28 to m/z 32 (O_2^+) will also be about 3, reflecting the composition of air. Under these circumstances, the leak should be located, fixed, and the repair documented. If the ratio of m/z 28 to m/z 18 is between 0.5 and 2, the situation should be monitored. Note that a large leak may result in saturation of the m/z 28 peak and give falsely low ratios. A noticeable increase in system pressure as well as the presence of significant amounts of m/z 14 (N^+) and m/z 16 (O^+) relative to m/z 18 are indications of a severe air leak.

Some manufacturers have elected to use the abundance of the m/z 28 ion from N_2 relative to m/z 69 from PFTBA tuning reference compound as an indication of an air leak. This approach is based on a consistent flow of PFTBA from the calibration gas vial. It is frequently specified in the range of 10 to 20%. The laboratory may choose to follow the manufacturer's recommendation on the specific relative abundance of these ions as a daily monitor for an air leak.

7.3 Calibration

7.3.1 Procedures

7.3.1.1 Single-Point Calibration

If the purpose of the analysis is to demonstrate that the concentration of a substance is greater than a threshold value, a single calibrator analyzed contemporaneously with a concentration equal to the threshold value may be used. Since the purpose of the measurement is to convey a degree of confidence that the value is greater than the threshold, it is recommended that the margin by which the sample exceeds the threshold be documented to have statistical significance.

7.3.1.2 Multipoint Calibration Curve

When a linear calibration curve is observed, the use of appropriate standard concentrations can maximize the precision of the curve at the threshold concentration. The narrowest point for the confidence band about a linear regression line occurs at the mean value of the ordinate (x) values. Where practical, concentrations for standards should be chosen so that the threshold concentration is at or near the mean value of the concentrations analyzed for the calibration.

The concentrations of the standards should be computed from the linear regression line and should agree within $\pm 20\%$ of the nominal value. If more than three concentrations are used to establish the curve, one standard may be dropped for not meeting acceptance criteria. Standards may not be dropped to improve the curve fit, to bring control results into acceptance range, or simply because its concentration is outside the target range. There must be a logical reason for dropping a standard (e.g., poor recovery, interfering peak, incorrect integration) and it must be documented.

7.3.1.3 Historical Multipoint Calibration Curve

If the standard curve has been documented to be linear and stable over a specified time interval, a historical calibration curve may be used to determine the concentration. In this case, at least two controls or standards should be analyzed (one of which should be at the threshold concentration), and the concentrations and ion ratios must be within $\pm 20\%$ of the initial measurements.

7.3.2 Internal Standard

It is advisable to use an internal standard, added prior to any extraction steps, to assess the performance of sample preparation and instrument function. For mass spectrometric detection, the use of a compound labeled with a stable isotope such as deuterium is recommended. If a deuterated internal standard is not available, or if a number of compounds are confirmed in the same procedure, it is acceptable to use a structurally related compound. The internal standard must undergo any derivatization reactions used in the procedure.

7.4 Quality Control/Quality Assurance

An important component of assuring analytical quality is an active quality assurance program. The program should encompass the range of compounds to be expected in the analysis, although the number of compounds confirmed and the number of analytical batches analyzed may preclude frequent assessment of every compound. The quality control samples must constitute at least 10% of the samples run in an analytical batch. Blind quality control samples should be included in the program which challenge not only the analytical portion of the assay, but also clerical and urine integrity testing if this is included as a part of the confirmation analysis.

Specific criteria must be established for acceptance or rejection of the quality assurance specimens. For quantitative assays, a comprehensive set of criteria, such as Westgard's rules, should be used if the volume of sample batches is sufficient to establish the precision characteristics of the assay. An acceptable alternative is to use $\pm 20\%$ of an established mean. Procedures should state the actions to be taken if quality assurance specimens fail to meet established criteria. Regular, active evaluation of quality assurance results must be documented.

Negative control samples should be routinely run, and must be negative in order to accept analytical batch results. It is recommended that a negative control sample be run immediately before each presumptively positive sample to ensure that there is no carryover from standards or controls.

If the confirmation procedure requires hydrolysis to remove glucuronide, sulfate, glutathione, or other conjugates, the hydrolysis procedure should be routinely assessed for completeness. It is recommended that the hydrolysis control, where available, be included with each analytical batch.

7.4.1 Specific Recommendations for Threshold Compounds

Quality assurance samples must be used near the analytical threshold. The concentration should be such that a positive finding is always achieved. A concentration 20 to 25% above the threshold is frequently used to achieve this goal. The positive control should be used for each threshold if more than one threshold is used in a single assay. A negative control with a concentration 20 to 25% below the threshold may be used to document the ability to determine a negative result near the threshold value. In cases where quantitative results are provided, a control sample may be used to document the linear range of the assay.

7.4.2 Specific Recommendations for Nonthreshold Compounds

Positive and negative control materials should be used in each analytical batch to document the presence and absence of the compound to be identified. It is recommended that the positive control contain a concentration comparable to that of the sample.

8 Identification Principles

In confirmation analyses, the objective is to identify the compound within a determined level of confidence. It has been suggested that the level of confidence for legal proceedings be 1 in 10,000 to 1 in 100,000.²⁸ Analysis procedures or combinations of procedures with greater information content increase the level of confidence in an identification.

8.1 Gas Chromatograph

Due to its high-peak resolution capacity, the use of capillary gas chromatography in conjunction with mass spectrometry significantly improves the information content of the analysis. It should be noted that co-elution of a peak with a compound of known structure does not prove identity, but rather demonstrates consistency with the identity of the known compound. The retention time of the peak due to the presumed compound should elute within $\pm 1\%$ or ± 0.2 minutes (whichever is smaller) of the retention time of a peak of a contemporaneously analyzed standard.

Chromatographic peak overload of matrix components or analytes may cause a shift in retention time. If a deuterated internal standard is used, the analysis may be accepted if the retention time difference between the internal standard and the proposed analyte is the same as the difference observed for a control or standard not exhibiting the peak overload condition. If no deuterated internal standard is used, standard addition of the suspected compound may be used to document co-elution. In this approach, an amount of pure standard comparable to that in the original analysis is added to the sample, and the sample is analyzed using the normal procedure. To document identity, only the chromatographic peak presumptively identified should be increased, and the peak profile at half-height should be within $\pm 10\%$ of the original peak width.

The peak of interest should be separated from any other peaks so that there is resolution of at least 1.25 or a valley of at least 90% between peak maxima (for equal-sized peaks). In GC/MS, the additional information obtained from mass-selective detection can be used to enhance chromatographic resolution. Deconvolution or peak purity algorithms can be used to resolve partially co-eluting peaks.²⁹ This can also be done manually by inspecting the consistency of mass spectra across the peak profile.

8.2 Mass Spectral Identification

A mass spectrum of an unknown component may provide a definitive identification. For this reason, application of GC/MS under appropriate conditions is considered the "gold standard" for identification. As mentioned above, acquisition of a complete mass spectrum is preferable to acquisition of selected ions. In those cases where high-quality full or partial spectra cannot be obtained, selected ion monitoring is acceptable.

8.2.1 Full-Scan Acquisition

The mass spectrum should be acquired from m/z 40 to at least 100 mass units above the expected molecular mass ion. In applications where ions from the derivatizing reagent dominate the spectrum, it is acceptable to acquire data from just above the mass of the derivatizing reagent. The acquisition of partial spectra may also be acceptable. In this case, the partial spectrum should be compared to that of a contemporaneously analyzed standard. Use of a spectral matching algorithm to compare a partial spectrum to a full spectrum is not acceptable.

Either the absence or presence of ions at a particular mass is informative. If manual inspection and identification of compounds is performed, the laboratory must develop guidelines for comparability of an unknown spectrum to that of a contemporaneously analyzed standard. Some typical guidelines may include: presence in the unknown spectrum of all mass ions with a relative abundance greater than 15% in the reference spectrum; agreement of ion intensities within $\pm 20\%$ relative abundance; absence of ions beyond the molecular ion cluster giving rise to a significant ion in spectrum (e.g., M-90 for TMS); absence of any ions greater than 50% relative abundance in the unknown spectrum that do not occur in the standard spectrum; relative abundance of isotopes in molecular ion cluster consistent with contemporaneous reference material or theory; and the absence of illogical mass losses in the spectrum. Inspection of the consistency of spectra across the peak profile may be helpful. The exercise of scientific expertise and judgment is appropriate.

8.2.2 Computer-Based Spectral Library Matching

The library search mode used in most laboratories is the identification search or reverse search. The search algorithm is designed to assess the presence of a target compound from a database of spectra in a spectrum obtained from a chromatographic peak. It is assumed in these algorithms that ions not in the reference spectrum are from impurities, and these ions are ignored. Other factors may also be applied to the experimental spectra in order to improve the ability of the algorithm to match a spectrum in the library database. The two most common commercially available algorithms are the probability-based matching (PBM) approach³⁰ and the dot product approach.^{b 31}

The reliability of computer-aided mass spectral matching to a library spectrum is dependent on a number of factors including: the search algorithm; the quality of the experimental spectrum; the presence of the spectrum in the library; the quality of the library spectra; the use of complete versus condensed library spectra; and instrumental factors such as tuning and source temperature. Decreasing analyte concentration makes all ions less abundant and more variable, decreasing the quality of the spectrum and the confidence index for the match.³⁰

The laboratory must establish criteria for acceptance of compound identification based on the "spectral match" quality. A spectral match factor of greater than 95 is generally associated with a correct identification. Since the match factor does not guarantee identification, all spectral library matches should

^b Distributed by the National Institute of Standards and Technology (NIST) and others.

be reviewed by a qualified scientist. It is recommended that for spectral match quality of between 75 and 95, the spectra should be evaluated by a qualified scientist for potential compound identification.

8.2.3 Selected Ion Monitoring Acquisition

As mentioned above, it has been demonstrated that the relative ion abundance ratios of three or four ions can be used to identify a compound. It should be noted that the ions must be diagnostic of the structure of the compound. In general, ions of higher abundance are selected due to their better reproducibility and lower limit of detection. Structurally significant ions should be selected over ions that have greater abundance but are not diagnostic. If sufficiently abundant, one of the ions selected should be the molecular ion (M^+). In any case, not more than one ion should be from any derivatization moiety.

There has been some confusion in the literature about the agreement between the observed ion ratios due to the fact that both the relative abundance and the variation in observed ratios have been described in percentage. At high relative abundance, this is of little consequence. For low relative abundance ions, there is a significant difference in outcome. For example, if a $\pm 20\%$ variation is computed at a relative abundance of 80%, a range of observed relative abundances between 64% and 96% would be accepted. The same criteria applied to an ion of relative abundance 10% results in an acceptable range of only 8 to 12%. If a $\pm 10\%$ abundance criteria is adopted, the corresponding ranges are 70 to 90% and 0 to 20%.

The laboratory must define what its identification criteria are for ion ratio matching. For the unknown compound, two ion ratios (three ions monitored) must be within the acceptable range for identification. The ion of largest abundance is frequently used for quantitative purposes and is referred to as the "quantification or quant ion" while the other two ions monitored for identification purposes are referred to as "qualifier or qual ions." For the internal standard, one ion ratio must be within the acceptable range (two ions monitored). It is recommended that the unknown compound have ion ratios within $\pm 20\%$ variation of the ion ratios measured in a contemporaneously analyzed standard if the relative abundance is greater than 20%, but a $\pm 5\%$ abundance range should be accepted when the measured relative abundance is less than 20% (e.g., $60 \pm 12\%$ and $18 \pm 5\%$ relative abundance). In addition, if the calculated acceptance range includes zero or a negative relative abundance, the lower end of the acceptance range should be 1% (i.e., the ion must be present).

The ion intensities for identification may be obtained from integrated peak height or peak area ratio measurements or from the ratios within a single acquisition. The ratio may be computed directly from the heights or areas. In order to be consistent with identity, all ions from the same peak profile must appear within one mass spectral acquisition of each other at the apex. If more than one standard is analyzed contemporaneously, the ion ratio acceptance range may be computed from a single standard, an average of standards, or a weighted average of standards. The method of computation must be documented. It is generally not acceptable to use different standards or different methods of computation for different specimens in the batch.

If screening procedure is also GC or GC/MS, and if less than three structurally characteristic ions are available, it is recommended that a second chemical technique or method be used. A change in derivatization chemistry can be considered a different technique.

8.2.4 Chemical Ionization

Chemical ionization mass spectra are characterized by less fragmentation but greater sensitivity than electron ionization. Since the ionization process is based on the kinetics of chemical reactions, the reproducibility of ion-relative abundances is somewhat smaller than for electron ionization. The specificity is dependent on the ionization conditions used and the uniqueness of the ions monitored. The ions produced from chemical ionization can be acquired in either the scan or SIM mode.

Acceptance criteria for compound identification with chemical ionization must be documented. The ion derived from the intact molecule [e.g., $(M+H)^+$ or $(M-H)^-$] or an ion closely related to the molecular species (e.g., loss of HF) should be monitored. Ion ratios compared to a standard run in the same batch should be within $\pm 25\%$ variation (e.g., for 80% relative abundance, acceptance range is 60 to 100%). Computation and evaluation of the ion ratios should be performed as described in Section 8.2.3. If full-scan acquisition is used, there should be no major ions present in the scan that are not derived from the proposed analyte.

8.2.5 Tandem Mass Spectrometry (MS/MS)

Tandem mass spectrometric methods may employ either electron or chemical ionization techniques. The fragmentation process is a physicochemical interaction, and as a result the ion intensity reproducibility is lower than that observed for electron ionization. The selective fragmentation of an ion of defined mass significantly increases the specificity of the technique. The ions produced from collision-induced fragmentation can be acquired in either the scan or SIM mode.

Acceptance criteria for compound identification with MS/MS must be documented. Collision conditions should be selected to ensure that the precursor ion is present in MS/MS scan. When monitoring one precursor to get one product ion, the resolution for the first mass analyzer should be set to unity (i.e. mass window equals one amu). If multiple ions are monitored for identification, ion ratios compared to a standard run in the same batch should be within $\pm 25\%$ variation (e.g., for 80% relative abundance, acceptance range is 60 to 100%). Computation and evaluation of the ion ratios should be performed as described in Section 8.2.3. If full-scan acquisition is used, there should be no major ions present in the scan that are not derived from the proposed analyte.

References

- ¹ Federal Register. 53 FR 11970. Mandatory Guidelines for Federal Workplace Drug Testing Programs: Final Guidelines Notice. 1988.
- ² Wu Chen NB, Cody JT, Garriott JC, Foltz RL, Peat MA, Schaffer MI. Report of 1988 Ad Hoc Committee on Forensic GC/MS: Recommended Guidelines for Forensic GC/MS Procedures in Toxicology Laboratories Associated with Offices of Medical Examiner and/or Coroners. *J Forensic Sci.* 1990;35:236-242.
- ³ Sphon JA. Use of mass spectrometry for confirmation of animal drug residues. *J Assoc Off Anal Chem.* 1978;61:1247-1252.
- ⁴ Baldwin R, Bethem RA, Boyd RK, et al. Limits to confirmation, quantitation, and detection. *J Am Soc Mass Spectrom.* 1997;8:1180-1190.
- ⁵ de Ruig WG, Dijkstra G, Stephany RW. Chemometric criteria for assessing the certainty of qualitative analytical methods. *Anal Chim Acta.* 1989;223:277-282.
- ⁶ Fetterolf DD, Yost RA. *Int J Mass Spectrom Ion Proc.* 1984;62(1);33-49.
- ⁷ *International Vocabulary of Basic and General Terms in Metrology.* 2nd ed. Geneva, Switzerland: International Organization for Standardization; 1993.
- ⁸ ISO/IEC Guide 2 General terms and their definitions concerning standardization and related activities. Geneva, Switzerland: International Organization for Standardization; 1996.
- ⁹ ISO/IEC Guide 25 General requirements for the competence of testing and calibration laboratories. Geneva, Switzerland: International Organization for Standardization; 1997.
- ¹⁰ IFCC/IUPAC: Approved recommendation (1978). Quantities and units in clinical chemistry. *Clin Chim Acta.* 1978;96:157F-183F.
- ¹¹ Price P. Standard definitions of terms relating to mass spectrometry: A report from the committee on measurements and standards of the American Society for Mass Spectrometry. *J Am Soc Mass Spectrom.* 1991;2:336-348.
- ¹² Duffus JH. Glossary for chemists of terms used in toxicology. *Pure and Appl Chem.* 1993;65:2003-2122.
- ¹³ Ettre LS. Nomenclature for chromatography. *Pure Appl Chem.* 1994;65:819-872.
- ¹⁴ David RB, Thompson JE, Pardue HL. Characteristics of statistical parameters used to interpret least square results. *Clin Chem.* 1978;24:611-620.
- ¹⁵ Kroll MH, Emmancipator K. A theoretical evaluation of linearity. *Clin Chem.* 1993;39:766-772.
- ¹⁶ Lipman HB, Astles JR. Estimating the linear analytic range. *Clin Chim Acta.* 199;282:15-34.
- ¹⁷ Currie LA. Nomenclature in evaluation of analytical methods, including detection and quantification capabilities (IUPAC Recommendations 1995). *Pure Appl Chem.* 1995;67:1699-1723.

- ¹⁸ Currie LA. Detection: Overview of historical, societal, and technical issues. In: *Detection in Analytical Chemistry: Importance, Theory, and Practice*. Washington, DC: American Chemical Society; 1988:1-62.
- ¹⁹ Hubaux A, Vos G. Decision and detection limits for linear calibration curves. *Anal Chem*. 1970; 42:849-855.
- ²⁰ Long GL, Winefordner JD. Limit of detection: A closer look at the IUPAC definition. *Anal Chem*. 1983;55:712A-724A.
- ²¹ Clayton CA, Hines JW, Elkins PD. Detection limits with specified assurance probabilities. *Anal Chem*. 1987;59:2506-2514.
- ²² Glaser JA, Foerst DL, McKee GS, Quave SA, Budde WL. Trace analysis for wastewaters. *Environ Sci Technol*. 1981;15:1426-1435.
- ²³ Lambert D, Peterson B, Terpenning I. Nondetects, detection limits, and the probability of detection. *J Am Stat Assoc*. 1991;266-277.
- ²⁴ Armbruster D, Tillman MD, Hubbs LM. Limit of detection (LOD)/limit of quantitation (LOQ): Comparison of the empirical and statistical methods exemplified with GC-MS assays of abused drugs. *Clin Chem*. 1994;40:1233-1238.
- ²⁵ Cleij P, Dijkstra A. Information theory applied to qualitative analysis. *Fr J Anal Chem*. 1979;298:97-109.
- ²⁶ Thieme D, Müller RK. Information theory and systematic toxicological analysis in "general unknown" poisoning cases. *Fr J Anal Chem*. 1997;358:785-792.
- ²⁷ Snyder LR, Kirkland JJ, Glajch JL. *Practical HPLC Method Development*. 2nd ed. New York: Wiley Interscience. 1997:714-720.
- ²⁸ de Ruig WG, Dijkstra G, Stephany RW. Chemometric criteria for assessing the certainty of qualitative analytical methods. *Anal Chim Acta*. 1989;223:277-282.
- ²⁹ Stein SE. An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectrometry data. *J Am Soc Mass Spectrom*. 1999;10:770-781.
- ³⁰ McLafferty FW, Hertel RH, Villwock RD. Probability based matching of mass spectra. *Org Mass Spectrom*. 1974;9:690-702.
- ³¹ Stein SE, Scott DR. Optimization and testing of mass spectral library searching algorithms for compound identification. *J Am Soc Mass Spectrom*. 1994;5:859-866.

Additional References

Boyd RK, Henion JD, Alexander M, et al. Mass spectrometry and good laboratory practice. *J Am Soc Mass Spectrom.* 1996;7:211-218.

Davis R, Frearson M, Prichard FE. *Analytical Chemistry by Open Learning, Mass Spectrometry.* New York: John Wiley & Sons; 1989.

Goldberger BA, Huestis MA, Wilkins DG. Commonly practiced quality control and quality assurance procedures for gas chromatography/mass spectrometry analysis in forensic urine drug-testing laboratories. *Forensic Sci Rev.* 1998;9:60-80.

International Conference on Harmonization. *ICH Harmonized Tripartite Guideline.* Adapted October 1994.

March RE, Todd JFJ, eds. *Practical Aspects of Ion Trap Mass Spectrometry.* Boca Raton: CRC Press. 1995:Vol. 1-3.

McLafferty FW, Turecek F. *Interpretation of Mass Spectra.* 4th ed. Mill Valley, CA: University Science Books; 1993.

Rood D. *A Practical Guide to the Care, Maintenance, and Troubleshooting of Capillary Gas Chromatography Systems.* Hüthig, Heidelberg; 1991.

Ullman MD, Bowers LD, Burtis CA. Chromatography/Mass Spectrometry. In: Burtis CA, Ashwood ER, eds. *Teitz Textbook of Clinical Chemistry.* 3rd ed. Philadelphia: Saunders. 1999:164-204.

NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

C43-P: Gas Chromatography/Mass Spectrometry (GC/MS) Confirmation of Drugs; Proposed Guideline

General

1. Consider including several figures that illustrate both a typical ion chromatograph and a total ion chromatograph.
 - **A figure has been placed at the end of Section 4.4.**
2. Overall I'd like to say the primary objective stated as to establish uniform practices for producing quality data for quantitation and identification of drug/metabolites was not met. To a practicing scientist, I don't believe that the explanations were specific enough. While most people have the impression that the SAMHSA guidelines are too strict and dictatorial, there is some comfort in knowing that interpretations are reproducible among labs following these criteria for their drug testing. When I read the title to this guideline, my first thought was great someone is finally going to set some standards in clinical and forensic medicine. However, I think specific examples for how to use these criteria in the clinical and forensic arenas are lacking.
 - **The SAMHSA guidelines are written in response to experience with the analysis of five compounds using primarily selected ion monitoring. It would be extremely difficult to cite examples for all of the possible scenarios for clinical and forensic testing. The commenter's opinion that the primary objective is not met, while appreciated, is not shared by the subcommittee.**

Foreword

3. Second paragraph, last sentence, "Appropriate application of these analytical tools requires that the methods are fit for their purpose and the instruments are operating correctly." This is somewhat vague. Are all the preceding methods mentioned in the context of being confirmatory methods? If so, perhaps the sentence should read 'Methods used are fit for the purpose of confirmation and....'
 - **The subcommittee believes that the sentence as written describes the situation accurately. Since the entire guideline applies only to confirmation analyses, the specifics of fit for purpose are determined by whether the compound must be quantified or identified.**

Introduction

4. In the Introduction, the statement "establish broader criteria" doesn't specifically say who the comparator is although it is inferred in the abstract that it is the SAMHSA guidelines.
 - **The wording has been changed to reflect that the criteria apply to areas other than drugs of abuse testing.**

Section 4

5. There are 5 pages of GC/MS instrument description. Scientists who are looking for guidelines involving GC/MS are probably quite familiar with the technique.
- **While this may be true, the experience of the subcommittee members is that significant amounts of incorrect information are circulated in the workplace. It is also likely that some scientists are not familiar with all of the techniques discussed. The subcommittee believes, therefore, that inclusion of this information establishes a uniform base upon which to build a guideline.**

Section 4.3.4

6. Section 4.3.4 touches on large amounts of air in the system. Section 7.2.2 helps identify ways to assess the system for air but nowhere is it included what instrument parts and parameters are damaged by air leaks.
- **There is specific reference to the damaging effects of air on the filament and column in Section 4.3.4. The deleterious effects on tuning are also indicated. The subcommittee therefore believes that this comment has been addressed.**

Section 6.1.1

7. For non-threshold compounds recommendation, should this include reference to whether these reference materials and metabolite isolates be run at the time of the unknown or run and stored as a database with which to compare the unknown spectrum? On the last line, it is unclear as to whether comparison of your unknown spectra or comparison of your reference materials to a reputable scientific journal is addressed here.
- **A sentence has been added to Section 6.1.1 to indicate that the secondary reference material should be analyzed contemporaneously with the unknown. It seems clear that the spectra obtained from the secondary reference material should be comparable to a spectrum published in the literature.**

Section 6.2

8. Second Paragraph: In this section on accuracy, it may be appropriate to reference CLIA '88 as one suggested interval for calibration verification to assess accuracy: according to CLIA it 'should be performed at least every 6 months and after major maintenance or change in reagents.'
- **A recommendation of at least every twelve months and after major maintenance has been made in Section 6.2.**

Section 6.2.1

9. Since carryover and contamination are the major sources of false positivity and inaccuracy in any high sensitivity measurements, the committee might consider strengthening Section 6.2.1 by including descriptions or examples of how to avoid or detect carryovers (e.g., negative runs...).
- **A sentence has been added to Section 6.2.1 using a blank injection as an example of a method to detect carryover.**

Section 6.4

10. Linearity, top paragraph. The discussion concerns choice of calibration concentrations for best precision about the regression line, rather than linearity per se. Perhaps a section should be added for calibration, or the heading for Section 6.4 could be changed to 'Calibration' with this paragraph moved first to precede discussion of linearity.
- **There is already a section on Calibration (Section 7.3). The subcommittee concludes that the inclusion of information on the assessment of linearity is best presented here.**
11. Linearity, first paragraph. If peak heights or areas (or ratios if an IS is used) are plotted as a function of analyte concentration to determine assay linearity, it should be clear that the approach to assess linearity must be the same as that used to obtain patient results.
- **Since the remainder of the section discusses the use of linear calibration to establish concentrations, this is implicit if not explicit in the discussion.**
12. Linearity, second paragraph. Correlation coefficient is a particularly poor way to assess assay linearity. It is not sensitive to deviations from linearity and the cut-off of 0.99 has no scientific basis. Reference should be made to NCCLS document EP6. For a discussion of the faults in using correlation coefficient, see Lipman and Astles, Clin Chim Acta, (1999) 282:15-34.
- **A revised paragraph discussing the limitations of correlation coefficient in determining linearity has been incorporated along with several additional references. A reference to NCCLS document EP6 was also added at the end of the section.**
13. Linearity, third paragraph. Authors may want to make the recommended frequency for checking linearity (currently yearly) to be the same as the frequency for checking calibration verification (Section 6.2).
- **The recommendation for linearity check has been changed to every twelve months.**

Section 6.5

14. It would be pertinent to include standard ways in which the industry is verifying LOD annually. Also, what specificity criteria should be applied to determining LOD for non-threshold compounds?
- **The third paragraph deals specifically with the current industry standard for verifying LOD.**

Section 6.6

15. It would be pertinent to include standard ways in which the industry is verifying LOQ annually.
- **The section deals specifically with the current industry standard for verifying LOQ.**

Section 7

16. Routine Instrument and Method Performance, second sentence. A statement is made that 'quality assurance samples' should be included with each batch. 'Quality control samples' would be more appropriate and that phrasing is used elsewhere (Section 7.4, first paragraph)."
- **The wording has been changed to "quality control samples."**

Section 7.1

17. Most people using GC do not even know what theoretical plates are let alone how to calculate them. It seems inappropriate to say they should calculate something we all know they probably will not. Perhaps something that they will actually do would be better.

The measurement of peak width at half height is something they can reasonably do. I think it should say something about ensuring the peak is represented in such a way that this measurement is possible. I have seen some peaks that are drawn on a wide time scale that the peak looks like nothing more than a spike, making the measurement impossible. Also, saying that time is acceptable to measure is reasonable, but distance seems inappropriate. Since the representation of a peak can be changed by telling the system how to draw it, as a result, distance can be satisfied by any peak. Measuring distance to determine asymmetry is reasonable. Perhaps I am not clear on what is meant by this paragraph.

- **Guidelines are intended to reflect good practice. If they can measure the peak width at half height, they can calculate theoretical plates.**

The purpose of measuring theoretical plates is twofold. First, since the width of the peak changes with the retention time (for an isothermal system), absolute width is not a good measure of anything. Second, since theoretical plates is a dimensionless ratio, the peak width and retention time can be measured in any units, so long as they are on the same scale (not expanding the scale for one and not the other). A definition of plate number has been included in Section 5 to assist in calculations.

18. Regarding the statement that "the rationale for recommendation of this resolution is the deviation is < 1% for...." Please reference this citation.

- **A reference has been added.**

Section 7.3

19. Section 7.3 doesn't address the differences in matrix found within the clinical and forensic practices.

- **A wide variety of matrices are found within clinical and forensic practice, and discussion of all of them is beyond the scope of this document.**

Section 7.4

20. Obtaining blind QC in these matrices presents the same problem. This also does not address how to make, how to store, and how to use these so-called quality control samples which should comprise 10% of an analytical batch.

- **The production of quality control materials is beyond the scope of this guideline. While the topic is of great interest, the strategies and limitations required for production of control materials in various matrices would require a separate document.**

Section 7.4.2

21. This might necessitate creation of a QC material with each confirmation run. While this practice is probably okay, there should be documentation that at some point the material used to create this QC or metabolite isolate was verified as to purity, etc. This criterion also suggests concentration matching

of a nonthreshold compound for which quantitation criteria have not been established and with no suggestion for how one might concentration match.

- **The production of quality control materials is beyond the scope of this guideline. While the topic is of great interest, the strategies and limitations required for production of control materials in various matrices would require a separate document.**

Section 7.4

22. It is not clear to me how a conjugated internal standard can be used to assess hydrolysis of a sample. This should be explained or I think it is something that people will not get right.

- **The subcommittee agrees and the last sentence of Section 7.4 has been deleted.**

Section 8.1

23. Most drug labs currently use $\pm 2\%$ for time. Is there any reason to set this to 1%? I have no real objection except we need to realize that this would make many labs change criteria. I do have serious concerns about using absolute retention time for differences. ± 0.2 minutes seems a problem for some long runs and unreasonably long for short retention time, particularly with the movement to shorter run times. As some drug testing labs work to get drug retention times down to 1-2 minutes, that criterion translates to $\pm 10\text{-}20\%$.

- **The purpose of this guideline is to address a broad spectrum of confirmation activities, not just drugs of abuse testing which is a high volume subset of the testing world. It would be appropriate to keep this broader spectrum of use in mind when discussing the guideline.**

That said, this is certainly a difficult issue. A $\pm 2\%$ interval at 20-minute retention time is ± 0.4 minutes; a 1% interval is ± 0.2 minutes which should be achievable. The subcommittee agreed that it should be possible to achieve 1% reproducibility in retention time. The phrase "whichever is smaller" was added to the first paragraph.

24. A valley of 20% is too high. Typically 10% is the most allowed. Why 20%? Also, there appears to be a discrepancy between this section and section 7.1. Also, it defines it as a valley assuming equal sized peaks, which seldom happens. There should be something said about unequal sized peaks, particularly when assessing the valley based on the peak of interest, not the largest (which many labs want to do).

- **The guideline now states that a resolution of 1.25 is required, which by definition is a 10% valley for equal sized peaks. The valley for a resolution of 1.25 changes with relative peak size, and for whether the peak of interest is the larger or the smaller peak. The point is that the use of a valley calculation is not recommended due to the changing situation with relative peak height. Additional discussion of the resolution issue is included in Section 7.1.**

Section 8.2.2

25. This section should give some guidelines about subtraction of background; not necessarily how to, but at least some things that are not appropriate.

- **The subcommittee believes this is beyond the scope of the document because there is not a standard approach which is varied between software programs.**

Section 8.2.3

26. At the end of the second to last paragraph discussion of not using different standards or different methods of computation for different specimens in a batch is discussed. While this is ideal, in the interest of time and cost, more than one presumptive positive may be included in a confirmation run. The article already discusses ways in which the concentration of a compound affects its identification and quantitation and adjustments that can be made to relative abundance and abundance criteria. I will say from experience that this is almost the rule and not the exception that specimens of differing concentrations, differing metabolites and differing ways in which individual bodies produce metabolites will be in the same batch confirmation run and will need essentially different identification criteria placed on them to determine positivity.

- **The comment, while relevant, does not indicate what the author would like the subcommittee to do.**

27. I get concerned with using absolute ion ratio ranges, particularly at the low end. Although I understand the problem with low abundance results, although my experience says it has more to do with low abundance than low relative abundance if the absolute abundance is high enough. No excuse for absolute ranges for urine drug testing except for a few compounds that are monitored at very low concentrations. Also paragraphs two and three give different suggestions of implementation. The first is particularly troublesome as I have inspected a lab that used absolute ranges that went from 0-20% for one of the PCP ions and guess what? 1% is inadequate in my view because that could well be background noise. To use such a criteria should be reserved only for rare and unique circumstances and not proposed as an option for most analyses.

- **The purpose of this guideline is to address a broad spectrum of confirmation activities, not just drugs of abuse testing, which is a high volume subset of the testing world. It would be appropriate to keep this broader spectrum of use in mind when discussing the guideline.**

This comment is somewhat difficult to understand, since by definition the mass spectrum is reported in relative abundance; thus the term “absolute abundance” is not clear.

Outside of the world of drugs of abuse, there are many examples of compounds that yield relatively few ions of relative abundance greater than 10%. Several publications have shown that in this case, the reproducibility of the ion signal from a mass spectrometer may not achieve $\pm 10\%$ — in essence, you are requiring better ion signal reproducibility for low intensity ions than you are for more intense ions.

The concept of using “0%” abundance for any ion makes little sense. This issue has been addressed in the third paragraph of Section 8.2.3.

Related NCCLS Publications*

- EP6-P2** **Evaluation of the Linearity of Quantitative Analytical Methods: A Statistical Approach; Proposed Guideline—Second Edition (2001).** This document provides guidelines for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.
- M29-A2** **Protection of Laboratory Workers from Occupationally Acquired Infections – Second Edition; Approved Guideline (2001).** This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of bloodborne infection from laboratory instruments and materials; and recommendations for the management of bloodborne exposure.
- NRSCL8-A** **Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).
- T/DM6-A** **Blood Alcohol Testing in the Clinical Laboratory; Approved Guideline (1997).** This document provides technical and administrative guidance on laboratory procedures related to blood alcohol testing, including specimen collection, methods of analysis, quality assurance, and reporting of results.
- T/DM8-A** **Urine Drug Testing in the Clinical Laboratory; Approved Guideline (1999).** This guideline addresses the development of procedures for analysis of urine to determine the presence of certain controlled substances; for specimen collection and processing; for methods of analysis; for quality assurance; and for the reporting and interpretation of results.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

NOTES

NCCLS ▼ 940 West Valley Road ▼ Suite 1400 ▼ Wayne, PA 19087 ▼ USA ▼ PHONE 610.688.0100
FAX 610.688.0700 ▼ E-MAIL: exoffice@nccls.org ▼ WEBSITE: www.nccls.org ▼ ISBN 1-56238-473-2





VIA OVERNIGHT MAIL and EMAIL

June 30, 2006

Joseph M. Papp
P. O. Box 329
Glen Spey, NY 12737
Joe.papp@mikefrayssesports.com

Re: Sample #941068 – International 42nd Presidential Cycling Tour of Turkey

Board of Directors

Ralph W. Hale, MD
Chair

Richard W. Cohen, MD
Vice Chair

Barry Axelrod
Treasurer

Kate Hendrickson Borg, CHMM
Secretary

Evelyn Ashford

Lawrence Brown, Jr., MD, MPH

Jean Fourcroy, MD, PhD, MPH

Andrew Mecca, Dr PH, MPH

Annette Salmeen, DPhil

Dear Mr. Papp:

Your urine sample collected at the International 42nd Presidential Cycling Tour of Turkey on May 7, 2006, was sent to the WADA accredited laboratory at Ankara, Turkey ("the Laboratory") for analysis. The Laboratory has reported that your A sample contains metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone), which are listed as prohibited substances in the class of anabolic androgenic steroids on the World Anti-Doping Agency's Prohibited List, adopted by both the USADA Protocol for Olympic Movement Testing ("Protocol") and the Union Cycliste Internationale ("UCI") Anti-Doping Rules. The Laboratory's positive A Sample report is enclosed with this letter. On June 27, 2006, USA Cycling requested USADA handle your positive case under the USADA Protocol.

At this time, in order to avoid delay in the adjudication of your case, you have the right to accept the Laboratory results. If you choose to accept the A Sample Laboratory results and do not wish to contest the finding of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone) in your sample, please sign the attached Acceptance of Laboratory Findings and Waiver Form and fax it to me at 719-785-2028 no later than July 10, 2006. Further, if you choose to accept the Laboratory results, we will forward your case immediately to a panel of the independent Anti-Doping Review Board, as set forth in the USADA Protocol, for its consideration. Under the USADA Protocol and the UCI Anti-Doping Rules, the finding of a prohibited substance or method in an athlete's sample constitutes a doping violation. If it is ultimately determined that this is your first doping violation, a sanction may be imposed that will include disqualification of any of your competitive results achieved from May 7, 2006, the day your sample was collected, and a two year period of ineligibility.

United States Anti-Doping Agency

1330 Quail Lake Loop, Suite 260, Colorado Springs, CO 80906 ■ Tel: 719.785.2000 ■ Fax: 719.785.2001

usada@usantidoping.org ■ www.usantidoping.org

GDC01359.1

If you choose not to accept the A Sample Laboratory results, your B Sample will be opened and analyzed at the Laboratory in Ankara, Turkey, on July 17, 2006. You and/or your representative have the right to be present at your expense to observe the B Sample opening and the analysis (which usually takes many hours in its entirety over multiple days). Please inform me in writing by fax at 719-785-2028 by July 10, 2006, if you plan to attend the B Sample analysis so that we may provide you with information on the time and address of the laboratory. Please contact me if you have any questions about the timing of the analysis. If you intend to compete in any protected competitions, USADA has the right under its Protocol, Section 13, to expedite this matter to final resolution prior to the protected competition.

Additionally, you have the right, at this time, to accept a "provisional suspension." By accepting a "provisional suspension," you will be immediately suspended from competing in all competitions under the jurisdiction of UCI, USA Cycling, and the United States Olympic Committee ("USOC"), until your case is deemed not to be a doping offense, you accept a sanction, you fail to contest this matter, or a hearing has been held in this matter. If you choose to accept this "provisional suspension," the time served under the "provisional suspension" will be deducted from any period of ineligibility that you might receive beginning on the date you accept the "provisional suspension" and notify USADA of such acceptance. If you choose not to accept this "provisional suspension," any period of ineligibility you might receive will begin on the date of your acceptance of the sanction or on the date of the arbitration hearing panel's decision.

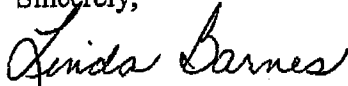
If you accept the "provisional suspension," USADA will give notice to the USOC, UCI, and USA Cycling of your acceptance of the "provisional suspension." Your decision to accept this "provisional suspension" is purely optional. You do not have to accept this "provisional suspension" in order to proceed with your case. **If you are willing to accept a "provisional suspension," please inform us in writing by July 10, 2006, by executing and returning the attached USADA Acceptance of Provisional Suspension Form.**

Also of importance, you are still subject to testing pending the outcome of this matter.

USADA will not publicly disclose or comment on the specifics of your test results until your case has been resolved. By copy of this letter, USADA is notifying USA Cycling and the USOC of your test results and requests that these organizations not comment publicly concerning this information until disclosed as provided in the USADA Protocol.

Enclosed for your reference are copies of the USADA Protocol and the World Anti-Doping Code, which set forth the administrative procedures followed for positive or elevated test results. You may also wish to contact John Ruger, the USOC Athlete Ombudsman who is completely independent of USADA, or your own personal attorney, for assistance or further information. Mr. Ruger may be reached at One Olympic Plaza, Colorado Springs, CO, 80909, by telephone at (888)-ATHLETE, by fax at (303) 444-6626 or by e-mail at John.Ruger@usoc.org, or at www.888athlete.org.

Sincerely,



Linda M. Barnes
Testing Results Manager

cc: Sean Petty, USA Cycling (w/o encls.)
Gary Johansen, USOC Deputy General Counsel (w/o encls.)
Jim Scherr, USOC (w/o encls.)

Enclosures: UCI Anti-Doping Control Test Certificate
Laboratory Certificate of Analysis
UCI Anti-Doping Rules
World Anti-Doping Code
WADA List of Prohibited Substances
USADA Protocol
USOC Anti-Doping Policies

UNITED STATES ANTI-DOPING AGENCY
ACCEPTANCE OF PROVISIONAL SUSPENSION

I, Joseph M. Papp, accept a "provisional suspension" as a result of the finding of the prohibited metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone) in my urine Sample #941068, collected at the International 42nd Presidential Cycling Tour of Turkey on May 7, 2006.

I understand and accept that I will not be able to compete in any competitions under the jurisdiction of the Union Cycliste Internationale ("UCI"), USA Cycling, or the United States Olympic Committee ("USOC") while serving this "provisional suspension."

I understand that the period of the "provisional suspension," beginning on the date I accept this "provisional suspension" and notify USADA of such, will be deducted from any period of ineligibility that I might receive in my case.

I understand and accept that USADA will notify UCI, USA Cycling, and the USOC of my acceptance of the "provisional suspension."

I understand and accept that my acceptance of the "provisional suspension" is purely voluntary and optional. I understand and accept that I am entitled to proceed with my case, to a hearing if necessary, regardless of whether I accept this "provisional suspension."

I understand and accept that I may serve this "provisional suspension" and it may ultimately be determined that no doping offense has occurred by the Panel of the USADA Anti-Doping Review Board or through a hearing.

I understand and accept that I am still subject to testing pending the outcome of this matter.

Signature of Joseph M. Papp

Date

Printed Name of Joseph M. Papp

UNITED STATES ANTI-DOPING AGENCY

ACCEPTANCE OF LABORATORY FINDINGS
WAIVER OF RIGHT TO B SAMPLE ANALYSIS AND
WAIVER OF RIGHT TO CONTEST LABORATORY FINDINGS

I, Joseph M. Papp, accept the finding of the WADA accredited laboratory at Ankara, Turkey (the "Laboratory") that my urine Sample #941068 collected on May 7, 2006 at the International 42nd Presidential Cycling Tour of Turkey contains the prohibited metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone). I understand that the Laboratory's findings are based on analysis of the A Sample of urine Sample #941068 and that the B Sample analysis on this Sample has not been conducted. I further understand that pursuant to the Protocol for Olympic Movement Testing of the United States Anti-Doping Agency, a sample shall not be considered positive until after the B Sample analysis confirms the A Sample analysis or the athlete has expressly waived the B Sample analysis. A waiver of the B Sample analysis means the sample shall be considered positive pursuant to the findings of the A Sample analysis. I have been advised of my right to have the B Sample analysis conducted and I voluntarily, knowingly, and intelligently waive my right to have a B Sample analysis conducted on Sample #941068. I do not contest the Laboratory's finding that metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH-androsterone) were in my urine sample. I voluntarily, knowingly, and intelligently accept the Laboratory findings and waive any right to contest the results of the Laboratory with respect to my Sample collected on May 7, 2006.

Signature of Joseph M. Papp

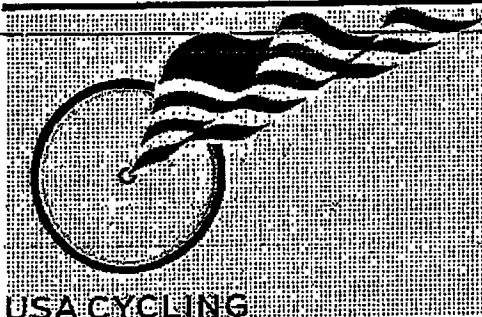
Printed Name of Joseph M. Papp

Date

Summary by USADA
Of Laboratory Documents
For Sample #941068

| | |
|-----------------------------|---|
| Sport: | Cycling |
| Sample Collection Date: | May 7, 2006 |
| Type of Collection: | In-Competition International 42 nd Presidential Cycling Tour of Turkey |
| WADA Accredited Laboratory: | Ankara, Turkey |
| Substance: | Metabolites of testosterone or its precursors (6 α -OH-androstenedione 6 β -OH- androsterone) |

This summary is based on the laboratory documents provided and is not intended to replace, substitute or in anyway supersede the laboratory documents. This summary is only for general reference purposes.



USA CYCLING

1 Olympic Plaza, Colorado Springs, CO 80909-5775

Facsimile Cover Sheet

To: Travis Tygart
Company: USADA
Phone:
Fax: 785-2028

From: Sean Petty
Company: USA Cycling
Phone: 719-866-4783
Fax: 719-866-4596

Date: June 26, 2006

**Pages including this
cover page:** 7

Comments:

Travis,
Attached is what we received from the UCI on File 22/06.

All the best,
Sean

26.JUN.2006 11:03

N2310 — P. 1 —



UNION CYCLISTE INTERNATIONALE

CH 1860 Aigle / Suisse

Tél. : +41 24 468 58 11 - Fax : +41 24 468 58 68 - e-mail : christian.varin@uci.ch

FAX MESSAGE

To : USA CYCLING
Mr. Steve JOHNSON
Fax nbr. : +1 719 866 46 28
From : Christian Varin, Manager
Copy : Agence Mondiale Antidopage
Mme Janie Soublière
+1 514 904 45 45
USADA
Mr. Tygart
+1 719 785 20 01
Date : June 26th 2006
Ref : Anti-Doping Services/ Cvd/DI
Total pages : 6 (including this one)
Subject : File 22/06

URGENT
CONFIDENTIEL

This facsimile may contain information that is confidential and which may be subject to legal privilege. If you are not the intended recipient, you must not pursue, use, disseminate or copy this message. If you received this message in error, please notify us by telephone (+41 24.468.58.11) and return the original message by mail. Thank you.

AVERTISSEMENT : Le contenu du présent fax ainsi que les documents qui y sont joints sont protégés par le secret professionnel. Toute communication, copie ou révélation de leur contenu à une personne autre que leur destinataire est strictement interdite et pénalement sanctionnée. Au cas où ce fax ne vous serait pas destiné nous vous remercions de bien vouloir nous en aviser immédiatement par téléphone (+41.24.468.58.11) et nous retourner l'original par courrier.

26.JUN.2006 11:03

N0310

P.2



INTERNATIONAL CYCLING UNION

Registered / Confidential

USA CYCLING

Mr. Steve JOHNSON

One Olympic Plaza

CO-80909 COLORADO SPRINGS

USA

Aigle, July 20th 2005

Ref: Antidoping / Cv / DI

File Nr. 22/06 (to mention in your correspondence)Joseph PAPP (licence 0027194 - UCI Code USA19750525) / Tour of Turkey (TUR), 7th May 2006

Dear Sir,

We inform you that the rider Joseph PAPP tested positive (OH-androstenedione, OH-androsterone) at the above-mentioned race according to the report we received from the Laboratory of Ankara (TUR), a copy of which we enclose (pursuant to article 187 of the Anti-Doping Rules). According to the laboratory's result on the A sample, the Anti-doping Commission must start from this notion that an offence against the mentioned Regulations has objectively been committed.

The rider is allowed to require without delay the B sample analysis (in accordance with articles 191 and following of the Anti-Doping Rules), or, failing that, it will be considered that he has renounced to this right. Mr. Joseph PAPP and/or his representative have also the right to attend the opening of the B sample and his analysis if this one is required. The rider is also allowed to require a copy of the complete analysis report for the samples A and B. In accordance with article 194 AR, to be acceptable the request for the analysis of the B sample shall be sent by the national federation no more than 5 working days after receipt of the letter informing it of the adverse analytical finding.

Please be reminded that, according to the Anti-doping Regulations - chapter X, the final sanction is foreseen by art. 261 and reads as follows:

Art. 261***Imposition of Ineligibility for Prohibited Substances and Prohibited Methods***

Except for the specified substances identified in article 262, the period of ineligibility imposed for a violation of article 15.1 (presence of Prohibited Substance or its Metabolites or Markers), article 15.2 (Use or Attempted Use of Prohibited Substance or Prohibited Method) and article 15.6 (Possession of Prohibited Substances and Methods) shall be:

26.JUN.2006 11:03

N0310

P.3



First violation: 2 (two) years' Ineligibility

Second violation: lifetime ineligibility

However, the License-Holder shall have the opportunity in each case, before a period of Ineligibility is imposed, to establish the basis for eliminating or reducing this sanction as provided in articles 264 and 265.

We would like to remind you that a violation of these Anti-Doping Rules in connection with an In-Competition test automatically leads to Disqualification of the individual result obtained in that Competition according to article 256. In addition, we also ask you to take into consideration the articles 257 to 260.

Regarding the cost of the proceedings, please refer to articles 244 to 246.

Moreover, we communicate you the text of the article 9.2.002 of the UCI Regulations which provides:

A rider against whom an investigation was opened in relation to a fact which may cause a breach of the UCI Anti-Doping Rules, will not be eligible for the World Championships until the end of the suspension or until his definitive acquittal. In the event of a positive A sample, this clause applies starting from the notification of the abnormal analysis result to the rider.

Unless otherwise decided by the anti-doping commission, the above paragraph is also applicable in the event of an investigation or a procedure regarding such a fact, opened in pursuance of a law or other regulation.

Specific cases are examined by the anti-doping commission or its president. Their decision is without appeal.

In addition to the disqualification, the licensee and his national federation will be respectively sanctioned by a fine of CHF 2000 to CHF 10000.

The present condition for participation, aims to protect the integrity, serenity and reputation of the World Championships. Its application does not prejudice the decision whether an anti-doping violation has occurred and shall not give rise to any claim in the events of acquittal.

We would consequently ask you to implement proceedings according to articles 188 to 205 of those Regulations. We would remind you that, according to the articles 249 to 251, you have to keep us informed of all measures you take by sending us copies thereof.

Looking forward to hearing from you.

Sincerely yours,

Antidoping Services,

Christian VARIN, Manager

Enc: mentioned

Copy to: WADA
USADA



26.JUN.2006 11:03

TURKISH DOPING CONTROL

03123052062 03123052262

N0310

P.4

HAZ. 13 2006 16:20 S1

KİŞİYE ÖZ
GİZLİTÜRKİYE
DOPING
KONTROL
MERKEZİ

Sayı: B.30.2.HAC.0.AP.00.00/2006-472

Türkiye Doping Kontrol Merkezi / Turkish Doping Control Center

29.05.2006

Analiz Sertifikası / Certificate of Analysis

Gençlik ve Spor Genel Müdürlüğüne,
Ulus/AnkaraCc: 1- WADA / Janis Soublière
2- BADNAG Başkanlığı
3- UCI

| No | Konu / Subject | Açıklama / Explanation |
|----|--|--|
| 1 | Federasyon-Spor / Federation-Sport | Bisiklet/ Cycling |
| 2 | Organizasyon / Event | Uluslararası 42. Cumhurbaşkanlığı Türkiye Bisiklet Turu / International 42th Presidential Cycling Tour of Turkey |
| 3 | Yarışma-Yarışma dışı / In Competition-Out of Competition | Yarışma / In competition |
| 4 | Sporcunun cinsiyeti / The gender of the athletes | 1 Erkek / 1 Male |
| 5 | Numunenin alınış tarihi ve yeri / Date and place of sample collected | 05-07.05.2006 - Antalya-Finike |
| 6 | Numunenin Merkeze geliş tarihi / Date of sample accepted | 09.05.2006 |
| 7 | Analize başlama ve bitiş tarihleri / Date of start and end of the analysis | 11.05.2006 / 25.05.2006 |
| 8 | Numunenin tipi (Kan/İdrar) / Type of the samples (Urine/Blood) | İdrar / Urine |
| 9 | Doping kontrol numune sayısı / Number of the samples | 1 (10 numunelik grubun içinden) / 1 (from a group of 10 samples) |
| 10 | Kayıt Numarası / Reception Batch | D2006RECE190 |

GİZLİ EVRAKTIR. İzinsiz paylaşılmaz.

1/2

Hacettepe Üniversitesi, 08100 Sıhhiye, Ankara
Tel: 0 312 810 67 76 Faks: 0 312 305 20 62 e-posta: td@mmsatur@hacettepe.edu.tr

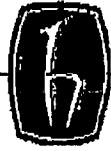
HEURE DE RECEPTION 13. JUN. 15:09

HEURE D'IMPRESSION 13. JUN. 15:10

GDC01359.11

26.JUN.2006 11:03
FROM : TURKISH DOPING CONTROL

03123052062 03123052062

N2310 P.5
HFZ. 13 2006 16:21 S2TÜRKİYE OZE!
GİZLİTÜRKİYE
DOPING
KONTROL
MERKEZİ

2436

| Lab. Kodu (A) / Lab. Code (A) | Şişe Kod Numarası (A) / Bottle Code (A) | WADA listesine göre numune de bulunan yasaklı madde ve/veya metaboliti ile kullanılan yasaklı yöntem / <i>Prohibited substances and/or their metabolites according to WADA found in urine</i> | Bildirilmesi gereken madde derişimi / <i>Urinary concentrations that must be reported</i> |
|---|---|---|---|
| D0601342 | 941068 | Metabolites of testosterone or precursors (Testosteron veya öncüsü metabolitleri) 6 α -OH-androstenedione 6 β -OH-androsterone | - |

Türkiye Doping Kontrol Merkezi'ne uygun koşullarda gelen yukarıdaki idrar numunesi(leri)nde, PROC1A, PROC2A, PROC4A, PROC4B, PROC5A, PROC6A ve PROC6B metotları kullanılarak gaz kromatografisi-kitle spektrometrisi ve immünoassay yöntemleri ile uyarıcı, narkotik, beta-blokör, anabolik ajan, kannabis metaboliti, diüretik, kokain metaboliti ve hCG grubu ilaçların tarama analizleri gerçekleştirilmiştir. Bu analizlerin sonucunda, yukarıdaki şişe kod numaralı sporcunun idrarında, Dünya Dopingile Mücadele Kurulu (WADA) tarafından yayınlanan "Dünya Dopingile Mücadele Yönetmeliği 2005 Yılı Yasaklı Maddeler Listesi Uluslararası Standardı" listesinde, yukarıda adı geçen yasaklı madde bulunmuştur.

Yukarıda adı geçen maddenin doğrulama analizi, gaz kromatografisi-kitle spektrometrisi ile gerçekleştirilmiştir.

The urine sample(s) received in good order above was (were) screened for stimulants, narcotics, beta-blockers, anabolic agents, metabolite of cannabis, diuretics, metabolite of cocaine and hCG with the methods PROC1A, PROC2A, PROC4A, PROC4B, PROC5A, PROC6A and PROC6B by using gas chromatography-mass spectrometry and immunoassay in Turkish Doping Control Center. After the analyses the substance above from the list of prohibited agents and methods issued by World Anti-Doping Agency (WADA) have been found.

The presence of the substance was confirmed in the sample by using gas chromatography-mass spectrometry.

Not: Pozitif numune sonucuna itiraz olduğu takdirde, bu rapor tarihinden (29.05.2006) itibaren en geç 3 hafta içerisinde merkezimize ile bağlantı kurularak analiz için uygun bir tarih belirlenmeli ve B numunesi analizi en geç 29.06.2006 tarihinde bitirilerek rapor edilmelidir. WADA kuralları gereği, bu tarih geçtikten sonra itirazda bulunulmuş olsun ya da olmasın, B numunesi analizi yapılmayacaktır.

Prof. Dr. M. Aytekin Temizer
WADA and ISO 17025 UKAS No: 2436 Accredited
Director of Turkish Doping Control Center

2/2

Hacettepe Üniversitesi, 06100 Sıhhiye, Ankara
Tel: 0 312 310 57 78 Faks: 0 312 305 20 52 e-posta: tdcm@hacettepe.edu.tr

HEURE DE RECEPTION 13. JUN. 15:09

HEURE D'IMPRESSION 13. JUN. 15:10

GDC01359.12

İzinsiz çoğaltılamaz.

GİZLİ DİR. İZİNİ VERİLMEZ.

26.JUN.2006 11:03 N0310 P.6
FROM : BSGM +90 312 3119637 HAZ. 20 2006 14:18 56



Union Cycliste Internationale



CONTRÔLE ANTIDOPAGE

ANTIDOPING CONTROL

Attestation du déroulement du contrôle

Test certificate

- Contrôle urinaire / Urine test ☒
1. En compétition / In competition ☒
Hors compétition / Out of competition ☐
3. Date: 08-05-2006
5. Course (nom, étape): International 42th Presidential
Race (name, stage): Cycling Tour of Turkey
7. Nom du coureur:
Surname of rider: PAPP

- Contrôle sanguin / Blood test ☐
2. Sexe: Masculin / Male ☒
Sex: Féminin / Female ☐
4. Lieu / Place: TURKEY - ALANYA
6. Discipline: ROAD
Discipline: ROAD
- Prénom: JOSEPH M
First name: JOSEPH M

Adresse du coureur:
Address of the rider: Via Hyacinthe Montebelli 26 51016 Montebelli Terme PT. Italia

9. Fédération nationale ayant délivré la licence:
National federation which delivered the license:

8. Code UCI:
UCI Code: USA 1975052-5

USA CYCLING FEDERATION

10. Numéro de licence:
License number: 0027194

11. Tiré au sort / Random: Oui / Yes ☐ Non / No ☒

12. Heure de présentation:
Presentation time: 13:15
Volume 10.5 cc pH 6.5
Optional / Optional

13. Heure de prélèvement:
Time of sampling: 13:35
Densité / Specific gravity: 1.015

14. Code des flacons:
Bottle codes: 341068 A+B

15. Heure de refus du coureur:
Time of rider's refusal: 14:30

16. Médicaments pris:
Pharmaceutical drugs taken by rider: MILKATIL ALFAXIL NOVALGIN
SINGULAR HYDROCOUSINE
C22W 1%

Contenu du livret de santé:
Contents of the health booklet:

17. Autorisation d'usage à des fins thérapeutiques (AUT):
Therapeutic use exemption (TUE) Oui / Yes ☒ Non / No ☐

18. Sauf les remarques ci-après, je confirme la régularité des opérations de prélèvement.
Subject to the comments below, I confirm that the sample was taken in accordance with the regulations.

Signature du coureur qui accuse également réception de sa copie:
Rider's signature who also acknowledges receipt of his copy: [Signature]

19. Accompagnateur:
Assistant: [Signature]

20. Médecin contrôleur:
Examining doctor: ENDEN Sizem
(nom/nome) (signature)

Agent de prélèvement sanguin:
Blood collection officer: [Signature]
(nom/nome) (signature)

21. Inspecteur antidopage:
Antidoping inspector: [Signature]
(nom/nome) (signature)

HEURE DE RECEPTION: 20. JUN. 14:08 HEURE D'IMPRESSION: 20. JUN. 14:13

HP LaserJet 4100 MFP



U.S. Anti-Doping Agency
7197852028
06/26/2006 05:06 PM

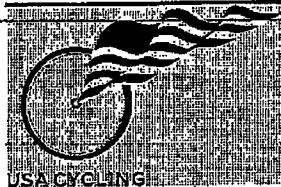
Fax Call Report

| Job | Date/Time | Type | Identification | Duration | Pages | Result |
|------|----------------|---------|----------------|----------|-------|--------|
| 1352 | 06/26 05:02 PM | Receive | 7198664596 | 03'50 | 7 | OK |

2006/TUE/26/MON 04:55 PM USA CYCLING

FAX No. 7198664596

P.001



1 Olympic Plaza, Colorado Springs, CO 80909-3775

Facsimile Cover Sheet

To: Travis Tygart
Company: USADA
Phone:
Fax: 785-2028

From: Sean Petty
Company: USA Cycling
Phone: 719-866-4783
Fax: 719-866-4596

Date: June 26, 2006
Pages including this
cover page: 7

Comments:

Travis,
Attached is what we received from the UCI on File 22/06.

All the best,
Sean

Catlin is leaving UCLA anti-doping lab

One of the world's leading authorities in sports doping, he is turning to full-time independent research.

By Michael A. Hiltzik, Times Staff Writer
March 14, 2007

Don H. Catlin, who founded UCLA's Olympic drug-testing laboratory in 1982 and built it into the busiest such facility in the world, said Tuesday he will retire from the university and turn to full-time research in sports doping.

"Basically, I know how to test," he said in a telephone interview from his Westside office, "but I don't have enough time anymore for my favorite avocation, which is research. Now I'll get to pick and choose my pet projects."

Catlin, 68, said those include developing a urine test for human growth hormone, which is reportedly growing in popularity as a sports doping substance, and improving the existing test for erythropoietin, or EPO, a performance-enhancing hormone that promotes the growth of red blood cells.

He will work through the Anti-Doping Research Institute, a non-profit lab he founded and that is located about a mile from the Olympic lab in West L.A. The institute, which is unaffiliated with UCLA, received a three-year, \$500,000 grant from Major League Baseball last year to develop an HGH test, which is currently undetectable in urine.

Catlin says the lab also has a commitment from the U.S. Anti-Doping Agency to provide it with a mass spectrometer, a crucial machine in drug testing, for a nominal lease fee, and is talking with the National Football League and other organizations to raise further funds.

"We're trying to get a handle on whether sport is really able and willing to support a research institute," he said.

As a professor at UCLA medical school, Catlin founded the Olympic lab at the request of the International Olympic Committee to provide drug testing at the 1984 Los Angeles Games. It is now part of a network of 34 anti-doping labs accredited by the World Anti-Doping Agency.

Of those, it is by far the busiest: Last year, the lab performed roughly 40,000 tests for anti-doping agencies, the NCAA, minor league baseball, and the NFL — nearly four times as many as the runner-up lab (in Cologne, Germany). The Olympic lab employs about 40 people.

One of the world's leading authorities in sports doping, Catlin's reputation is closely tied to the lab, and it is unclear whether the facility will retain its prominence after his departure. A spokesman for UCLA said the university hopes to keep the lab operating as part of its David Geffen School of Medicine. In a statement issued Tuesday, the Geffen school's dean, Gerald S. Levey, said he would "immediately begin discussions" with WADA and the other lab clients "with respect to the continuation" of the lab's association with the school.

Catlin said the transition to a new lab director, yet to be selected, would take about a month, and that he would become an emeritus professor at the Geffen school, where he plans to continue teaching.

As lab director, Catlin notched numerous milestones in the field of sports doping, notably his identification in 2003 of tetrahydrogestrinone (THG) a "designer steroid" illicitly dispensed to athletes by the notorious Bay Area Laboratory Cooperative, or BALCO.

But he has also been critical of the existing anti-doping system, which he believes is underfunded by sports and anti-doping organizations and unduly fixated on proving athletes dirty. Instead, he has proposed giving athletes an incentive to establish their cleanliness by volunteering for long-term medical profiling.

Under WADA rules, Catlin has been barred from offering his expertise as a witness to athletes defending themselves against anti-doping charges. He said Tuesday that he was unsure under what circumstances he would take on such assignments as an independent researcher.

"I'm not going to be out there testifying against my old friends," he said. "But I'll be out there trying to get it right for sport, for the agencies, for the athletes. It has to be right for everybody."

Catlin is leaving drug-testing lab at UCLA

One of the world's leading authorities in sports doping, he is turning to full-time independent research.

By MICHAEL A. HILTZIK
Times Staff Writer

Don H. Catlin, who founded UCLA's Olympic drug-testing laboratory in 1982 and built it into the busiest such facility in the world, said Tuesday he will retire from the university and turn to full-time research in sports doping.

"Basically, I know how to test," he said in a telephone interview from his Westside office, "but I don't have enough time anymore for my favorite avocation, which is research. Now I'll get to pick and choose my pet projects."

Catlin, 68, said those include developing a urine test for human growth hormone, which is reportedly growing in popularity as a sports doping substance, and improving the existing test for erythropoietin, or EPO, a perform-

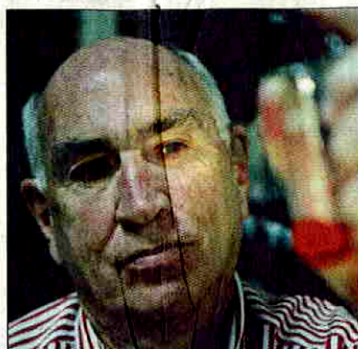
ance-enhancing hormone that promotes the growth of red blood cells.

He will work through the Anti-Doping Research Institute, a non-profit lab he founded and that is located about a mile from the Olympic lab in West L.A. The institute, which is unaffiliated with UCLA, received a three-year, \$500,000 grant from Major League Baseball last year to develop an HGH test, which is currently undetectable in urine.

Catlin says the lab also has a commitment from the U.S. Anti-Doping Agency to provide it with a mass spectrometer, a crucial machine in drug testing, for a nominal lease fee, and is talking with the National Football League and other organizations to raise further funds.

"We're trying to get a handle on whether sport is really able and willing to support a research institute," he said.

As a professor at UCLA medical school, Catlin founded the Olympic lab at the request of the International Olympic Committee to provide drug testing at the 1984 Los Angeles Games. It is now part of a network of 34 anti-



ALLEN J. SCHABEN Los Angeles Times

PIONEER: As director of the Olympic drug-testing lab at UCLA, Don H. Catlin notched numerous milestones in the field of sports doping.

doping labs accredited by the World Anti-Doping Agency.

Of those, it is by far the busiest: Last year, the lab performed roughly 40,000 tests for anti-doping agencies, the NCAA, minor league baseball, and the

NFL — nearly four times as many as the runner-up lab (in Cologne, Germany). The Olympic lab employs about 40 people.

One of the world's leading authorities in sports doping, Catlin's reputation is closely tied to the lab, and it is unclear whether the facility will retain its prominence after his departure. A spokesman for UCLA said the university hopes to keep the lab operating as part of its David Geffen School of Medicine. In a statement issued Tuesday, the Geffen school's dean, Gerald S. Levey, said he would "immediately begin discussions" with WADA and the other lab clients "with respect to the continuation" of the lab's association with the school.

Catlin said the transition to a new lab director, yet to be selected, would take about a month, and that he would become an emeritus professor at the Geffen school, where he plans to continue teaching.

As lab director, Catlin notched numerous milestones in the field of sports doping, notably his identification in

2003 of tetrahydrogestrinone (THG) a "designer steroid" illicitly dispensed to athletes by the notorious Bay Area Laboratory Cooperative, or BALCO.

But he has also been critical of the existing anti-doping system, which he believes is underfunded by sports and anti-doping organizations and unduly fixated on proving athletes dirty. Instead, he has proposed giving athletes an incentive to establish their cleanliness by volunteering for long-term medical profiling.

Under WADA rules, Catlin has been barred from offering his expertise as a witness to athletes defending themselves against anti-doping charges. He said Tuesday that he was unsure under what circumstances he would take on such assignments as an independent researcher.

"I'm not going to be out there testifying against my old friends," he said. "But I'll be out there trying to get it right for sport, for the agencies, for the athletes. It has to be right for everybody."

michael.hiltzik@latimes.com



CONFIDENTIAL

UCLA OLYMPIC ANALYTICAL LABORATORY
DEPARTMENT OF PHARMACOLOGY
UCLA SCHOOL OF MEDICINE
2122 GRANVILLE AVENUE
LOS ANGELES, CALIFORNIA 90025
PHONE: 310-825-2635
FAX: 310-206-9077

July 10, 2006

Terrence P. Madden
United States Anti-Doping Agency
1330 Quail Lake Loop, Suite 260
Colorado Springs, CO 80906-4651

RE: Specimen number USADA [REDACTED], Site ID OOC = [REDACTED]

Dear Mr. Madden:

Please find enclosed the documentation package for the case identified above.

Enclosed are authentic photocopies of the original documentation supporting our conclusion and the drug testing report.

Please feel free to call if you have any questions.

Sincerely,

A handwritten signature in cursive script, appearing to read "Michael Sekera".

Michael Sekera
Certifying Scientist

cc: Don H. Catlin

CONFIDENTIAL
DOCUMENTATION

SAMPLE IDENTIFICATION:

Organization requesting test: USADA
Date of sample collection: May 28, 2006
Site ID: OOC
USADA Sample Code Number: [REDACTED]
UCLA Code: [REDACTED]
Substances identified: T/E ratio greater than four

TABLE OF CONTENTS

"A" SAMPLE CONFIRMATION DOCUMENTATION

Page

GC/IRMS Data

Negative urine QC

Chromatogram of ion 44..... 3

Table of $\delta^{13}\text{C}$ values..... 3

Positive calibrator (unextracted androsterone and etiocholanolone standards)

Chromatogram of ion 44..... 4

Table of $\delta^{13}\text{C}$ values..... 4

Sample urine aliquot

Chromatogram of ion 44..... 5

Table of $\delta^{13}\text{C}$ values..... 5

TMS derivative, SIM GC-MS data, IS = Ethylmorphine

Negative urine QC

Extracted ion chromatogram of Testosterone, Epitestosterone and IS..... 6

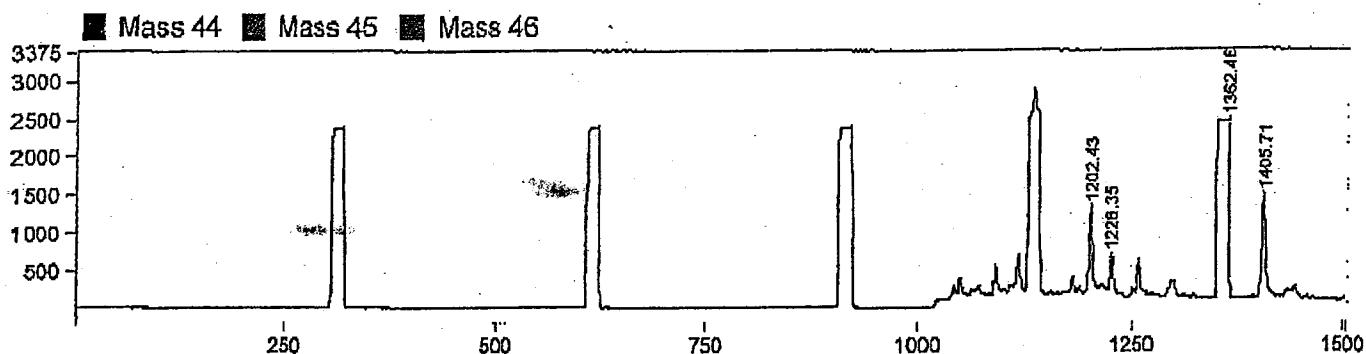
Positive calibrator (unextracted testosterone and epitestosterone standards at a ratio of 4:1)

Extracted ion chromatogram of Testosterone, Epitestosterone and IS..... 7

Sample urine aliquot

Extracted ion chromatogram of Testosterone, Epitestosterone and IS..... 8

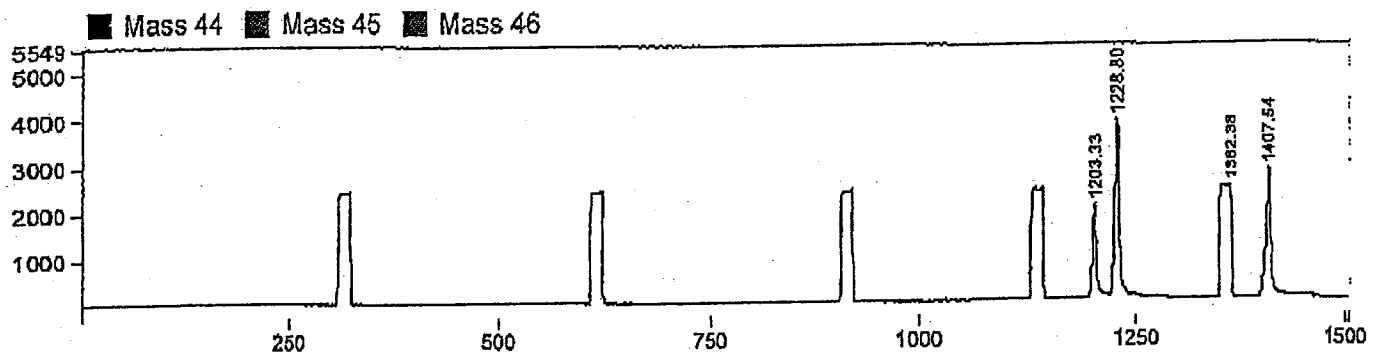
| | AS S | AS Method | Identifier 1 | Comment | Preparation | Post Script | Method |
|---|------|----------------|--------------|---------|-------------|-------------|--------------------|
| X | 48 | >Internal No 9 | NEGQC#5 | | | | method1[diois].met |



| Rt [s] | δ 13C/12C [per mil] vs. VPDB |
|--------|-------------------------------------|
| 1202.4 | -26.084 |
| 1226.3 | -27.427 |
| 1362.3 | -25.862 |
| 1405.7 | -25.862 |

BA 7/7/06

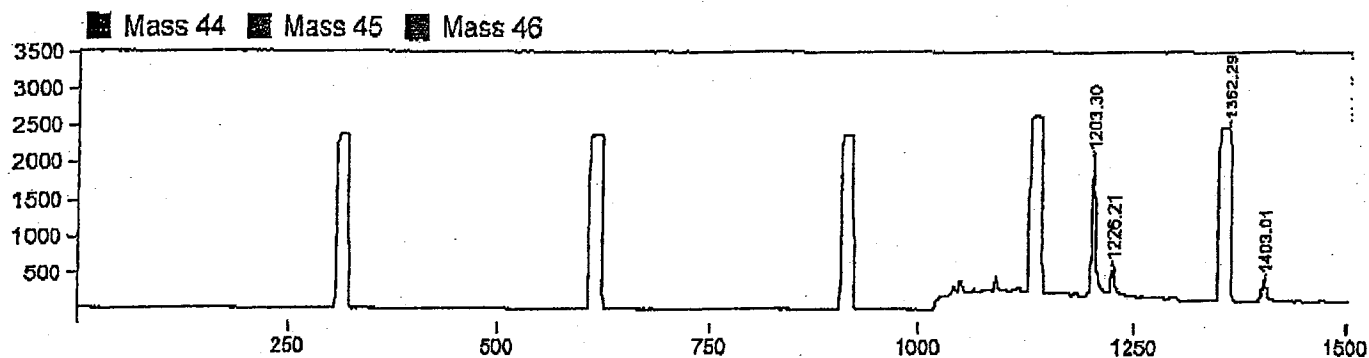
| AS S | AS Method | Identifier 1 | Comment | Preparation | Post Script | Method |
|------|-----------|----------------|----------|-------------|-------------|--------------------|
| X | 46 | >Internal No 9 | Diol STD | | | method1[diols].met |



| Rt [s] | d 13C/12C [per mil] vs. VPDB |
|--------|------------------------------|
| 1203.3 | -37.658 |
| 1228.8 | -36.640 |
| 1382.4 | -33.510 |
| 1407.5 | -23.130 |

BA 7/7/06

| AS | AS S | AS Method | Identifier 1 | Comment | Preparation | Post Script | Method |
|----|------|----------------|--------------|---------|-------------|-------------|--------------------|
| X | 52 | >Internal No 9 | | | 10 mL | | method1[diols].met |



| Rt [s] | d 13C/12C [per mil] vs. VPDB |
|-----------|------------------------------------|
| 1203.3 | -32.736 |
| 1226.2 | -31.737 |
| 1362.3 | -33.540 |
| 1403.0 | -25.417 |

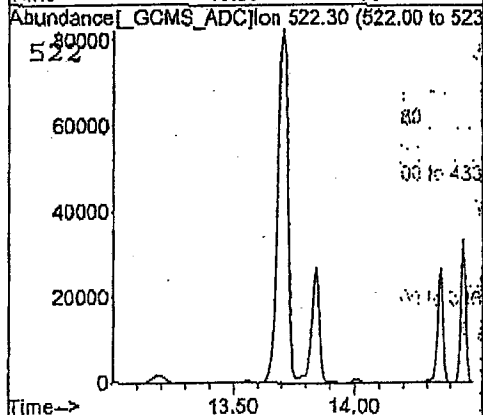
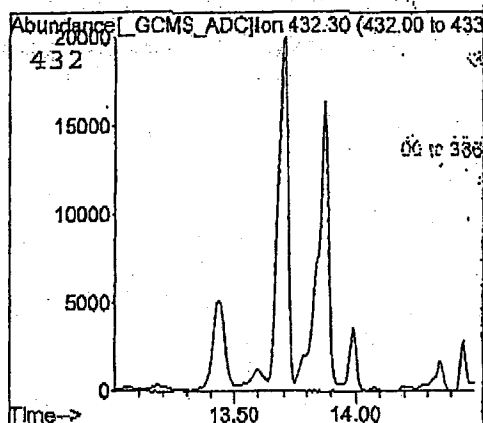
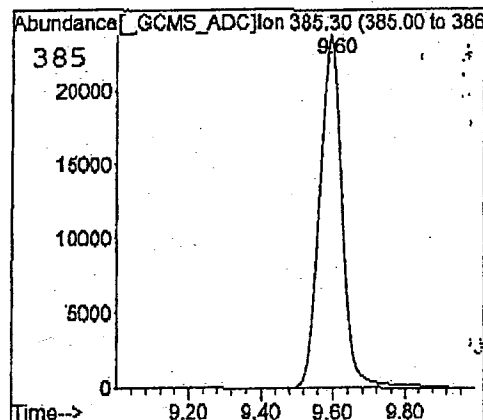
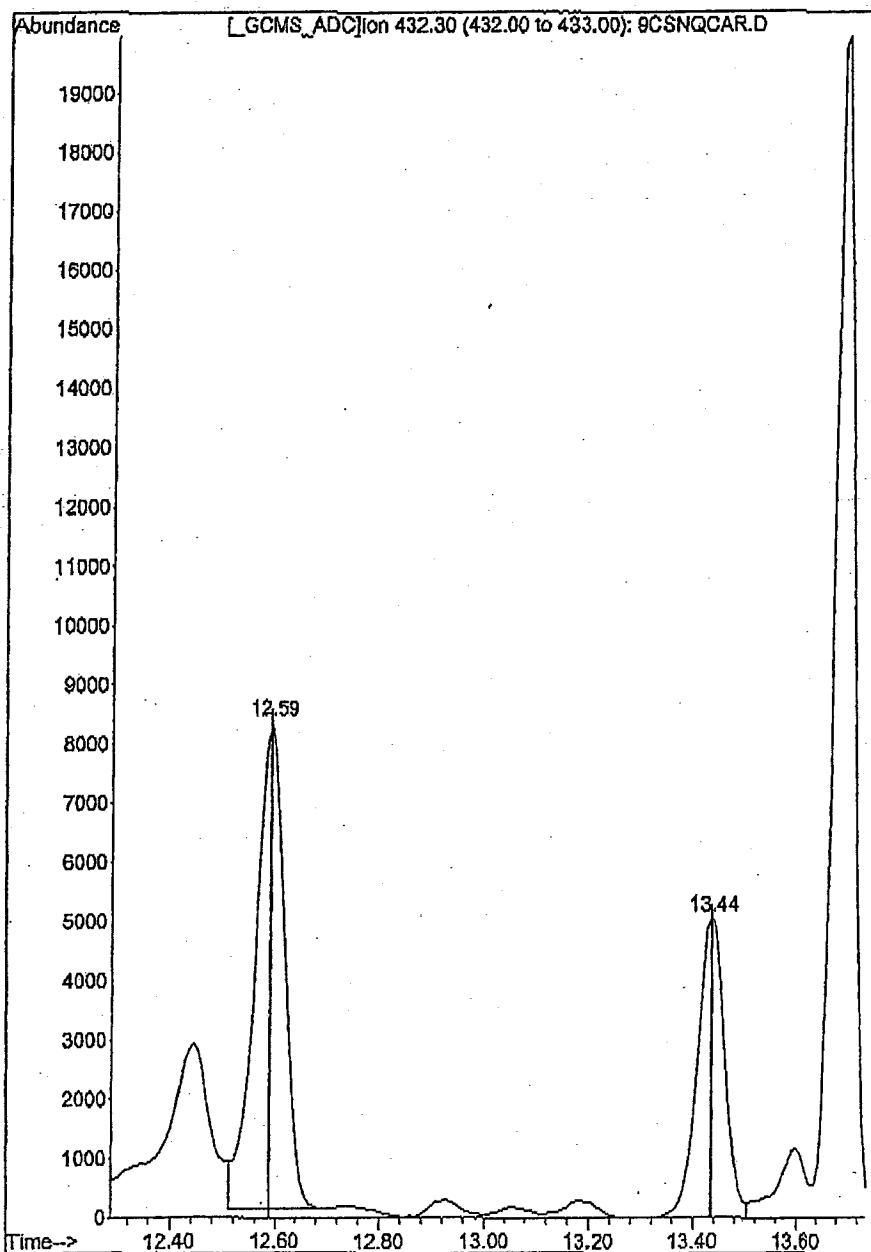
UCLA OLYMPIC ANALYTICAL LABORATORY

p 6

Sample Name : TE NEGATIVE QC
Data File : 9CSNQCAR.D
Miscellaneous:
Equipment # : MSDA13
Method File : TESIM04A
Analysis Time: 29 Jun 2006 1:43 pm
ALS Bottle # : 73

| | RT | RRT | PEAK HEIGHT |
|-------------------------|--------|------|-------------|
| Internal Standard (385) | 9.595 | 1.00 | 23767 |
| Testosterone (432) | 13.435 | 1.40 | 5097 |
| Epitestosterone (432) | 12.589 | 1.31 | 8407 |

Testosterone/Epitestosterone Peak Height Ratio = 0.606



11/30/06

1/30/06

GDC01362.6

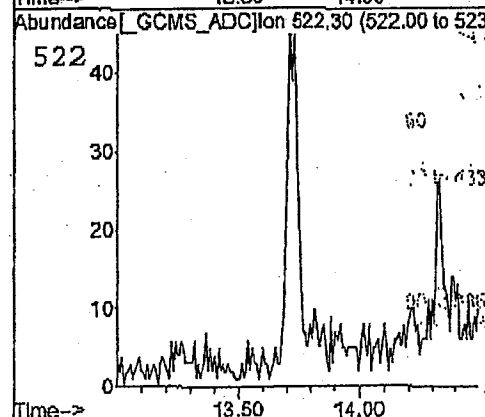
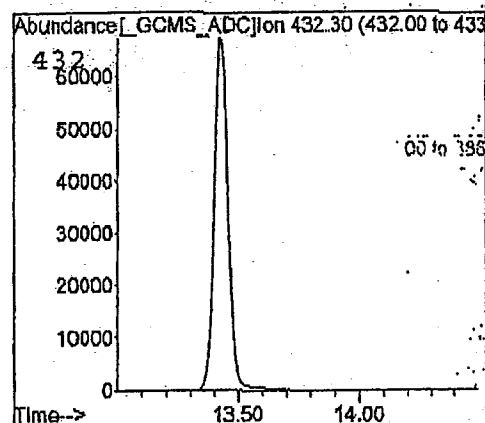
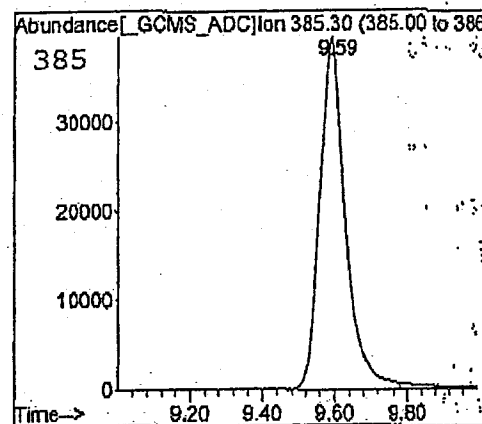
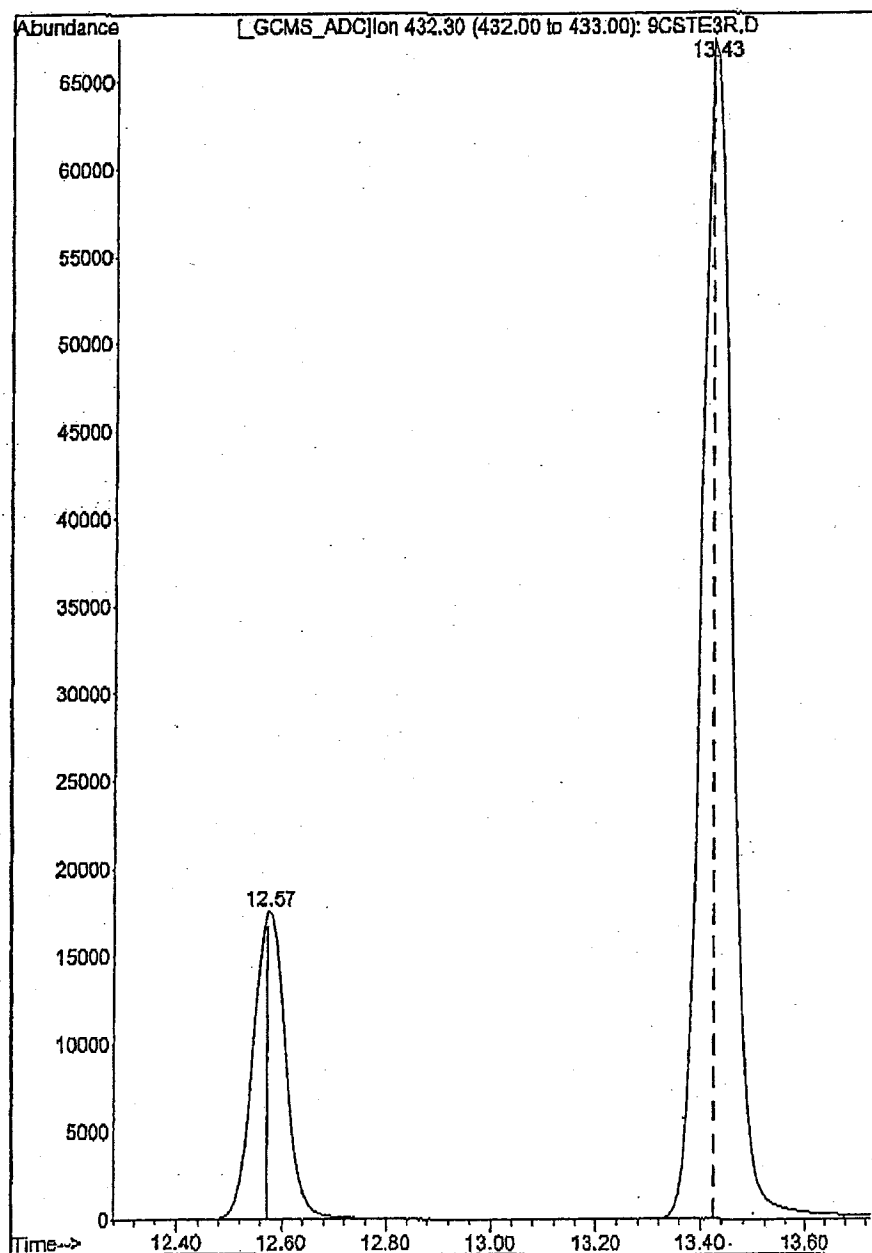
UCLA OLYMPIC ANALYTICAL LABORATORY

p 7

Sample Name : TE 4:1 STANDARD
 Data File : 9CSTE3R.D
 Miscellaneous:
 Equipment # : MSDA13
 Method File : TESIM04A
 Analysis Time: 29 Jun 2006 8:15 pm
 ALS Bottle # : 86

| | RT | RRT | PEAK HEIGHT |
|-------------------------|--------|------|-------------|
| Internal Standard (385) | 9.594 | 1.00 | 39563 |
| Testosterone (432) | 13.427 | 1.40 | 67499 |
| Epitestosterone (432) | 12.572 | 1.31 | 17643 |

Testosterone/Epitestosterone Peak Height Ratio = 3.826



Mel/30/02 6/30/02 DC01362.7

UCLA OLYMPIC ANALYTICAL LABORATORY

Sample Name : 9C E A CONFIRMATION
 Data File : 9Cb
 Miscellaneous:
 Equipment # : MSDA13
 Method File :
 Analysis Time: 4:21 pm
 ALS Bottle # : 79

p 8

| | RT | RRT | PEAK HEIGHT |
|-------------------------|--------|------|-------------|
| Internal Standard (385) | 9.594 | 1.00 | 28498 |
| Testosterone (432) | 13.444 | 1.40 | 60727 |
| Epitestosterone (432) | 12.589 | 1.31 | 1081 |

Testosterone/Epitestosterone Peak Height Ratio = 56.177

